Short communication

Improved selectivity for high-performance liquid chromatographic determination of clonazepam in plasma of epileptic patients

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Abstract

We report a high-performance liquid chromatography method for clonazepam determination in plasma. The use of a synthetic silica-based stationary phase markedly improved clonazepam resolution compared to standard reversed-phase columns. A liquid–liquid extraction was used, associated with reversed-phase chromatography, gradient elution and ultraviolet detection. Accuracy and precision were satisfactory at therapeutic concentrations. Selectivity was studied for benzodiazepines or other antiepileptic drugs, with particular attention to newly marketed drugs i.e., gabapentine and vigabatrin. No interfering substance was evidenced. Under the conditions described, it was possible to quantify clonazepam at nanogram level even when carbamazepine was present at therapeutic concentrations. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Clonazepam is a benzodiazepine derivative primarily used in the treatment of epilepsy. It has been shown to be effective in various types of seizures in both adults and children [1]. Its use is based on daily administration of an oral dose to prevent seizures or intravenous infusions in status epilepticus [2] and neonatal convulsions [3]. In both cases, monitoring plasma concentrations helps to adjust dosage.

Effective clonazepam plasma concentrations are low and methods for quantitative determination must have the required sensitivity. The range generally accepted as therapeutic is 15–80 ng ml^{-1} at predose sampling [3,4]. Moreover, there is a risk of increased seizure frequency when clonazepam plasma con-centrations exceed 120 ng ml^{-1} [5]. Achieving a low threshold of quantification is therefore essential for a quantification method to be applied to small volume samples such as in pediatric patients.

Further difficulty in monitoring plasma clonazepam concentrations results from frequent multiple therapy, and particularly the interference from carbamazepine. Carbamazepine concentrations are 100- to 500-fold greater and the poor selectivity of most high-performance liquid chromatography (HPLC) assays limits correct measurement of clonazepam when carbamazepine is co-administered. Furthermore, two drugs have recently been marketed in France (gabapentine and vigabatrin) and their potential interference must be studied.

The first published methods for quantitative determination of clonazepam for therapeutic monitoring were based on gas chromatography with electron

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capture detection [6,7]. During the last 20 years, numerous liquid chromatography assays have been described [8–11] that are suitable for therapeutic drug monitoring. Most of them involve solvent extraction and in some cases solid-phase extraction [8]. Chromatographic separation is generally performed on a reversed-phase octadecyl column with effluent monitoring by ultraviolet detection. Improvements in sensitivity and selectivity have been achieved by different means involving optimised sample preparation (i.e., back-extraction), use of large sample volumes (up to 2 ml) or amine mobile phase modifiers such as butylamine to avoid peak broadening.

Clonazepam is a basic drug that is not well resolved by classic reversed-phase columns. Until recently we used a classic octadecyl silane column, similar to those described elsewhere. In certain cases, we were unable to quantify clonazepam because of low concentrations or interference from carbamazepine. The availability of a new kind of stationary phase designed to avoid asymmetric peaks with basic compounds was therefore promising and evaluation of its performance was undertaken. The present paper describes the improvement obtained by using this column packed with a synthetic silica phase.

2. Experimental

2.1. Materials

Clonazepam was kindly supplied by Roche Laboratories (Neuilly sur Seine, France). Tetrazepam, used as internal standard, was obtained as a pure substance from Sanofi-Pharma (Gentilly, France). The distilled water used in buffers and eluents was purchased from Aguettant (Lyon, France). Acetonitrile and dichloromethane (Merck, Darmastadt, Germany) were of HPLC grade and potassium dihydrogenphosphate and sodium hydroxide (Sigma, Saint-Quentin-Fallavier, France) of analytical grade.

2.2. Calibration

Stock solutions of clonazepam (1 mg ml^{-1}) and tetrazepam (1 mg ml^{-1}) were prepared by dissolving 10 mg of pure compound in 10 ml of acetonitrile.

These solutions were stored for three months at 4°C in glass bottles. Working solutions were prepared each day by making appropriate dilutions of the stock solutions in acetonitrile.

A separate stock solution was used to prepare quality control specimens by spiking human drug-free plasma at concentrations of 5 and 50 ng ml^{-1}. The same batch of control samples was used during the entire study period and stored at -20°C.

Six plasma calibration standards (2.5, 5, 10, 25, 75, 100 ng ml^{-1}) were prepared for each analytical run by spiking 1 ml of blank plasma with 100 μl of the appropriate clonazepam solution. The same amount (100 μl) of pure acetonitrile was added to the blank, quality controls and unknown specimens.

2.3. Sample preparation

Each calibration standard and plasma sample or quality control were supplemented with 500 ng of tetrazepam and two drops of a 2 *M* NaOH solution to give a final pH of 10. The mixture was shaken for 5 min with 10 ml of dichloromethane. After centrifugation for 5 min, the organic layer was transferred into glass centrifuge tubes. The solvent was evaporated to dryness under a stream of nitrogen at 60°C and reconstituted in 40 μl of acetonitrile, 20 μl of which was injected onto the chromatograph.

2.4. Instrumentation

The HPLC system consisted of a Waters 600 multisolvent delivery system for gradient elution and a Waters 486 variable-wavelength absorbance detector (Waters Associates, Saint-Quentin-en-Yvelines, France). System management and data acquisition were performed by the Waters Millennium 2010 software implanted on a NEC 466 personal computer.

2.5. Chromatography

Separation was performed on a Symmetry C$_{18}$ (250×4.6 mm) column (WAT 054225) equipped with a guard column packed with the same material (WAT 054275). The mobile phase consisted of a gradient of acetonitrile and 20 m*M* phosphate buffer.

The latter was prepared by dissolving 1.09 g of KH_2PO_4 in 400 ml of distilled water. pH was adjusted to 7 with NaOH to prevent column alteration at higher pH values. Components of the mobile phase were filtered separately through a 0.45-mm PTFE membrane filter (Millipore, Saint-Quentin-en-Yvelines, France) and degassed by bubbling helium. The flow-rate was 1.0 ml min^{-1}. A linear gradient from 50 to 70% acetonitrile was applied for 15 min. The system then returned to initial conditions and was equilibrated for 5 min between each injection.

Detection was carried out at 313 nm and peak area ratios of clonazepam to internal standard were plotted against concentrations to construct calibration curves.

2.6. Validation procedures

Method validation was conducted according to recently published guidelines [12].

The recovery of clonazepam from plasma was assessed by adding a known amount of clonazepam to blank plasma to give a concentration of 50 ng ml^{-1}. Extraction efficiency was calculated by comparing the peak areas obtained from spiked plasma with those from injected pure standards.

Assay performance was evaluated according to intra- and inter-day accuracy and precision, determined from replicate analysis of control specimens at 5 and 50 ng ml^{-1}. These were calculated with commonly accepted procedures. Intra-assay accuracy and precision were examined by analysing five replicates at each concentration and inter-assay reproducibility was determined on five separate occasions.

Results were considered satisfactory when accuracy and precision were within +15% of the actual value, except for the limit of quantitation where it should not exceed 20%.

Selectivity was evaluated for other antiepileptic drugs: clobazam, nor-clobazam, diazepam, nor-diazepam, phenobarbital, primidone, phenytoin, carbamazepine, valproic acid, progabide, vigabatrin, gabapentine and ethosuccimide were injected as pure substances under the assay conditions. Selectivity was considered satisfactory when resolution values (R_s) were >2.

3. Results and discussion

The major problem responsible for peak broadening with silica-based column packing is undesirable silanol interactions [13]. A high-purity synthetic silica has recently been marketed to resolve these problems encountered with basic compounds. This product appeared to meet the criteria for improved resolution for clonazepam monitoring in epileptic patients.

3.1. Chromatography

Fig. 1 shows a typical chromatogram obtained from plasma spiked with clonazepam (75 ng ml^{-1}) and tetrazepam (500 ng ml^{-1}). Clonazepam had a retention time of 6.4 min and tetrazepam of 15.1 min.

3.2. Column lifetime

It is generally recommended in the literature that a guard column be used to protect the analytical column and extend its life. In our experience, the performance of the Symmetry column decreased over time if the guard column was omitted and we subsequently replaced it every 200 injections. After each run, the column was washed with deionized water for at least 1 h to prevent precipitation of the buffer and then left in 100% acetonitrile overnight and for prolonged storage.

3.3. Selectivity

Elimination of interference is critical for quantification in the low-nanogram range of clonazepam therapeutic concentrations. UV detection was set at 313 nm to minimize absorbance from biological substances and to avoid interference from other drugs. Under these conditions, coadministered drugs are less likely to produce interference responsible for measurement errors.

Clonazepam and carbamazepine were well resolved (retention times: 6.4 min and 4.7 min, respectively). Use of a gradient is necessary when carbamazepine is present at a therapeutic level that produces a large peak in comparison with clonazepam. Under these conditions, resolution of the two drugs was
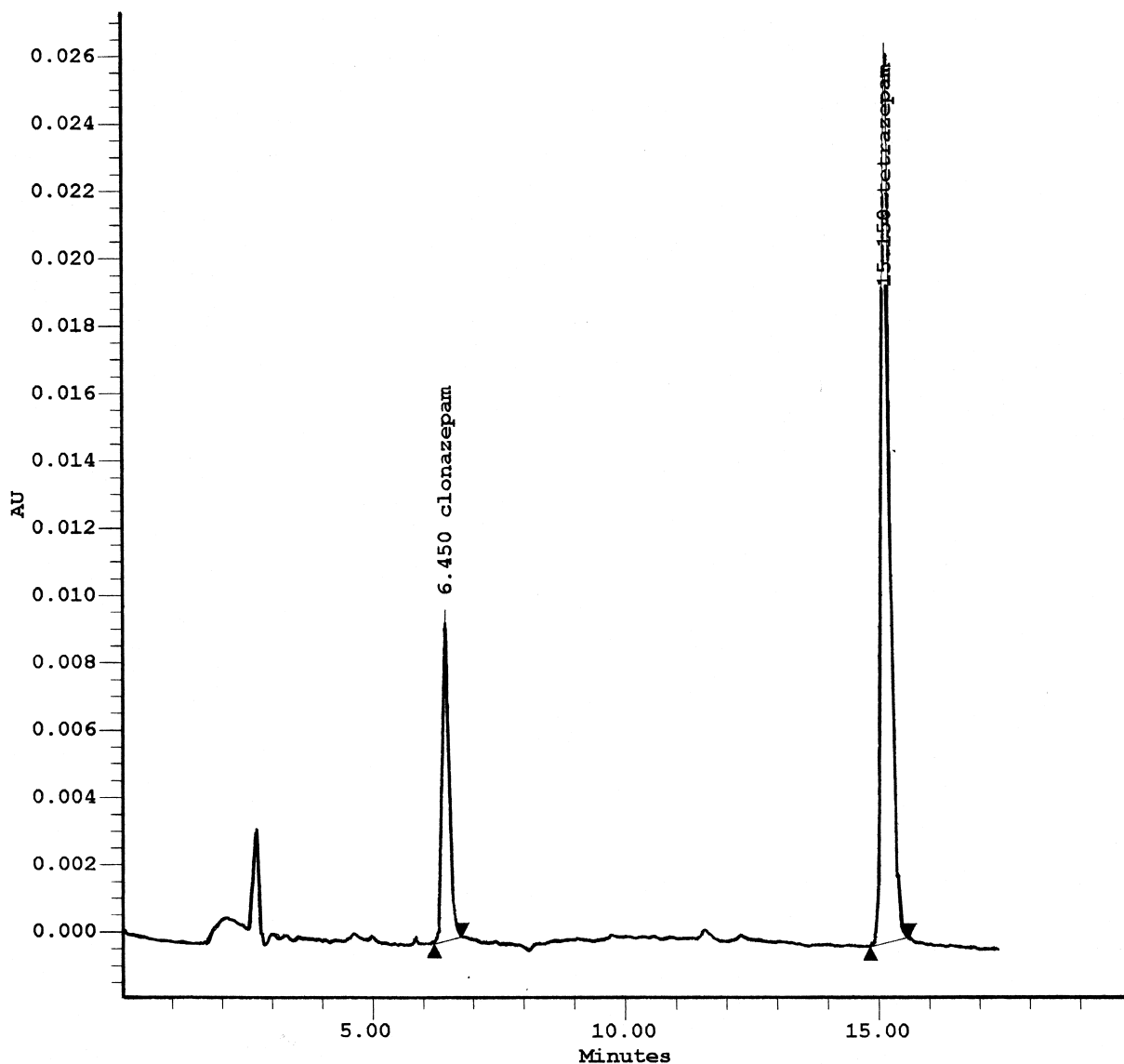
Fig. 1. Chromatogram obtained after extraction of a blank plasma spiked with 75 ng ml^{-1} clonazepam and 500 ng ml^{-1} tetrazepam.

excellent (R_S=3.25) and we were able to quantify clonazepam even when carbamazepine was coadministered. Fig. 2 shows a chromatogram obtained from a patient treated with clonazepam (28.5 ng ml^{-1}) and carbamazepine (9.4 μg ml^{-1}).

The retention times of other antiepileptic drugs tested for possible interference are presented in Table 1. None of the drugs tested interfered with clonazepam assay. Some drugs produced well re-

solved peaks which were distinguishable from clonazepam or internal standard. Others such as vigabatrin or gabapentine were not detected.

Tetrazepam was selected as internal standard because it was the last benzodiazepine eluted among those tested, thus preventing delay between each injection to avoid interference of the previous chromatogram in cases of benzodiazepine intake. Nevertheless, the use of tetrazepam as internal standard is
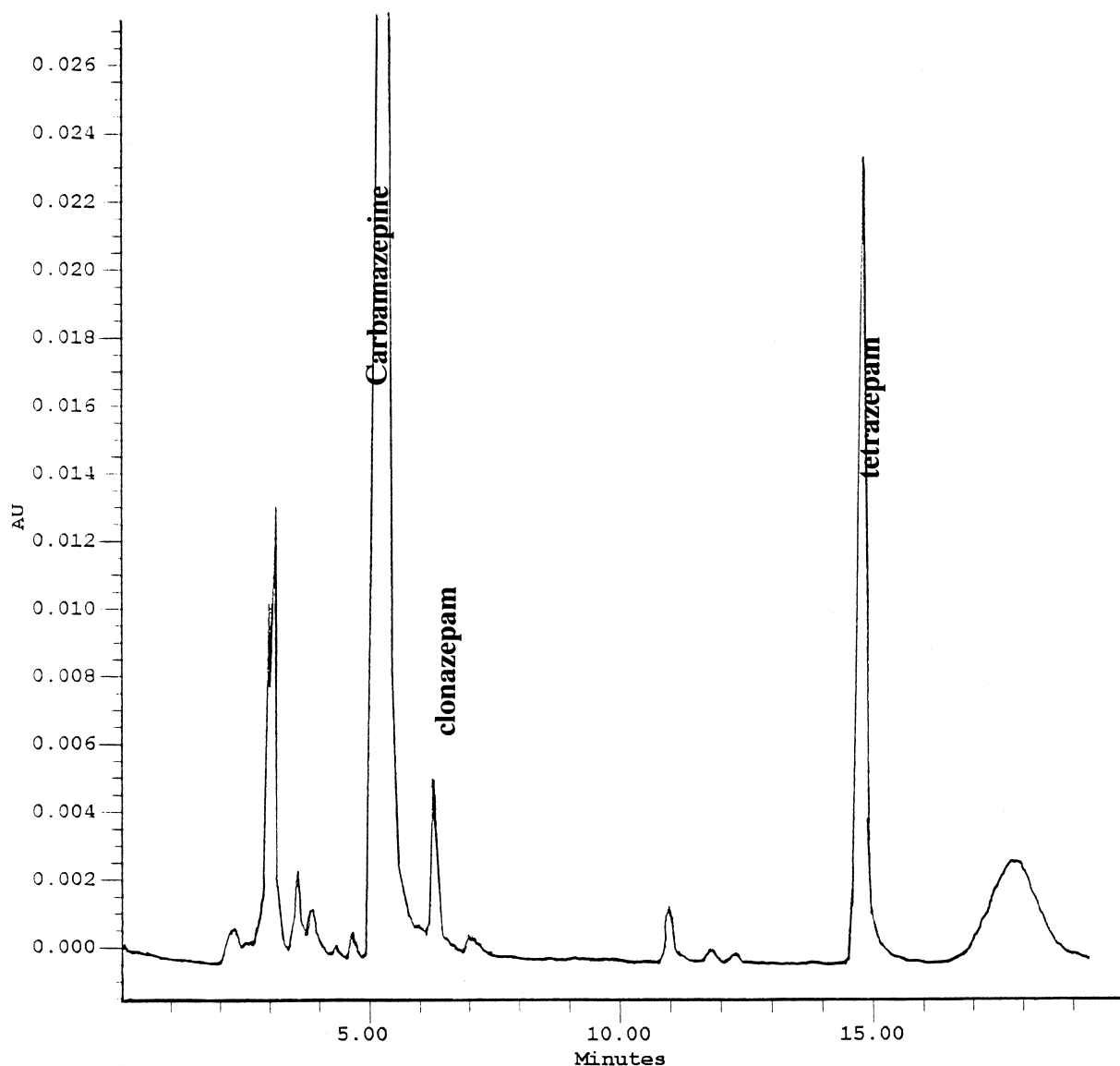
Fig. 2. Chromatogram from a patient treated with clonazepam and carbamazepine. Clonazepam concentration was 28.5 ng ml^{-1} and that of carbamazepine 9.4 mg ml^{-1}.

restricted in cases of its concomitant prescription. In such cases, another benzodiazepine derivative should be used.

3.4. Extraction procedure

The mean recovery of clonazepam (50 ng ml^{-1}) from plasma averaged 101% ($n=5$). The extraction procedure was simple and we did not try to optimise its selectivity. Although all basic and lipophilic drugs are likely to be extracted, attempts to avoid such interference would involve back-extraction. This has been proposed by some authors [12]. In our case, the chromatographic resolution of clonazepam from endogenous peaks compensated for these problems. No interfering peak was observed in the blank

Table 1
Selectivity of the assay for other antiepileptic drugs

Compound	Retention time (min)
Carbamazepine	4.70
Clobazam	9.30
Clonazepam	6.20
Diazepam	10.4
Ethosuccimide	3.92
Gabapentine	N.D.
Norclobazam	3.25
Nordiazepam	7.60
Phenobarbital	N.D.
Phenytoïn	N.D.
Primidone	N.D.
Progabide	9.20
Tetrazepam	15.1
Valproic acid	N.D.
Vigabatrin	N.D.

N.D.=Not detected by the assay.

sample, as confirmed by the mean intercept value of the calibration curves which were close to zero.

3.5. Limit of detection and linearity

The limit of detection (LOD), corresponding to the lowest concentration that produces a peak height approximately three-times the baseline noise, is 2 ng ml^{-1}. This high sensitivity is due to sharper peaks, resulting from the great improvement in chromatographic resolution.

Calibration curves were established on each day of analysis. The relationship between peak area ratio and clonazepam concentration in the range of 2.5 to 100 ng ml^{-1} was linear [$y=0.011$ ($\pm1.06\cdot10^{-3}$)$x+1.9\cdot10^{-3}$ ($\pm1.8\cdot10^{-4}$); $r=0.9998$) with a relative standard deviation (R.S.D.) for the slope of 9.5% ($n=10$).

3.6. Accuracy and precision

Data concerning intra- and inter-assay accuracy and precision were determined as described in Section 2.6. Tables 2 and 3 present results obtained as means, accuracy and R.S.D. The limit of quantification was 5 ng ml^{-1} as it was the lowest tested concentration at which both accuracy and precision were within the proposed criteria. Such a limit is adequate for routine therapeutic drug monitoring. However, given the high sensitivity of this assay, achieving a lower limit of quantification seems possible.

3.7. Application

The present method is used routinely to determine the plasma concentrations of clonazepam in adults and children. To date, 21 samples have been analyzed, 20% of them corresponding to subjects receiving carbamazepine as concomitant therapy. Clonazepam concentrations ranged from 7 to 55 ng

Table 2
Intra-day accuracy and precision for clonazepam in spiked human plasma

Clonazepam added (ng ml^{-1})	Mean found (ng ml^{-1}, $n=5$)	Accuracy[a] (%)	Precision (R.S.D., %)
5	5.65	13.0	12.4
50	53.5	7.0	7.6

[a] Accuracy: concentration found expressed in % of the nominal concentration.

Table 3
Inter-day accuracy and precision for clonazepam in spiked human plasma

Clonazepam added (ng ml^{-1})	Mean found (ng ml^{-1}, $n=5$)	Accuracy[a] (%)	Precision (R.S.D., %)
5	5.45	9.0	11.7
50	49.1	1.8	10.8

[a] Accuracy: concentration found expressed in % of the nominal concentration.

ml^{-1} with a median value of 11 ng ml^{-1}. Three patients had clonazepam concentrations <5 ng ml^{-1}.

4. Conclusions

This HPLC method provides a marked improvement for clonazepam measurement in plasma. Without a complex chromatographic procedure, clonazepam can be quantified at nanogram level even with small volume samples or concomitant carbamazepine administration. This method is suitable for therapeutic drug monitoring and pharmacokinetic studies.

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