Journal of Chromatography, 341 (1985) 383—390
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2584

LIQUID CHROMATOGRAPHIC ANALYSIS OF CLONAZEPAM IN HUMAN SERUM WITH SOLID-PHASE (BOND-ELUT®) EXTRACTION

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(First received November 15th, 1984; revised manuscript received February 7th, 1985)

SUMMARY

A simple, sensitive, selective and precise liquid-column chromatographic assay for clonazepam is described, in which 1 ml of serum containing 50 μg/l methylclonazepam as an internal standard is extracted by elution from a Bond-Elut® column with 400 μl of methanol. An aliquot of the eluate is injected on to a reversed-phase column and eluted with a mobile phase of acetonitrile—phosphate buffer (30:70) at a flow-rate of 2 ml/min at a column temperature of 50°C. Detection is at 254 nm. Chromatography is complete in 12 min. A sensitivity of 2 ng/ml is attained when 1 ml of serum is extracted. Analytical recovery of the clonazepam added to serum ranged from 91% to 99% with a coefficient of variation of 6.0%. This assay for clonazepam has good precision, with coefficients of variation of 11% at 15 ng/ml and 2.6% at 50 ng/ml. There was no interference from any of the commonly used antiepileptics.

INTRODUCTION

Clonazepam or 5-(2-chlorophenyl)-3-dihydro-7-nitro-1,4-benzodiazepine-2-one is a close structural and pharmacological analogue of nitrazepam. It has been effectively used in the treatment of petit mal epilepsy and minor motor seizures of childhood, and in refractory grand mal epilepsy, focal motor seizures, temporal lobe epilepsy, myoclonic epilepsy, and by intravenous route, in status epilepticus. It is usually used in patients who are already resistant to other antiepileptic drugs [1].

A thin-layer chromatographic (TLC) method for the assay of clonazepam was reported by Wad and Hanifl [2]. Since then, modifications of this method have been described [3]. However, the sensitivity of this method limits its usefulness to heavy overdose situation. Radioimmunoassay methods for clonazepam suffers [4—7] from cross-reactivity of clonazepam antibody with its 7-amino

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and 7-acetylamino metabolites. Currently gas—liquid chromatographic (GLC) procedures using electron-capture detection [8—12] of either the unchanged drug [8, 12—15] or derivatives [16—20] are widely used for the assay of clonazepam. The unchanged drug produces poor response with electron-capture detection, hence the need for acid hydrolysis and/or derivatization of the drug. The laborious extraction procedures often required for the isolation of the drug from plasma, chemical manipulation and long retention times make GLC methods very time-consuming. There are relatively few liquid chromatographic (LC) methods published for the analysis of clonazepam [21—23]. A normal-phase separation for clonazepam was reported by Perchalsky and Wilder [21] using dihydrodiazepam as internal standard. However, clonazepam and carbamazepine are not well resolved under normal-phase conditions. Revel and Sanjuan [23] used reversed-phase LC to separate clonazepam from carbamazepine but could not resolve chlordiazepoxide from clonazepam. Jambor [24] reported good recovery of clonazepam with chloroform extraction. However, the internal standard, dihydrodiazepam, was found to be unstable.

A liquid-column chromatographic method which obviates these drawbacks and offers considerable improvement in speed, accuracy, and selectivity in the monitoring of clonazepam is described here. The solid-phase extraction procedure used in this method is very simple, does not utilize large amounts of organic solvents, and ten samples can be processed in about 15 min. Both chlordiazepoxide and carbamazepine are well resolved from clonazepam in this assay.

EXPERIMENTAL

Chromatography

The analysis was carried out on a Series 3B (Perkin-Elmer, Norwalk, CT, U.S.A.) liquid chromatograph equipped with a Model 7125 (Rheodyne, Cotati, CA, U.S.A.) injector, an LC-15 fixed-wavelength ultra-violet detector (Perkin-Elmer), a 10-mV recorder Model BD 40 (E and K Scientific Products, Saratoga, CA, U.S.A.) and a 15 cm × 4.6 mm I.D. reversed-phase column (Altex, subsidiary of Beckman Instruments, Berkeley, CA, U.S.A.) packed with Ultrasphere ODS C_{18}, 5 μm particle size. The column was mounted in an LC 100 (Perkin-Elmer) oven maintained at 50°C. The column was eluted with acetonitrile—0.02 mol/l phosphate buffer, pH 3.8 (30:70) at a flow-rate of 2.0 ml/min, and the column effluent was monitored at 254 nm.

Extraction apparatus

C_{18} Bond-Elut® columns and a Vac-Elut® apparatus were obtained from Analytichem (Harbor City, CA, U.S.A.).

Reagents and standards

All reagents were of reagent-grade purity. All inorganic reagents were made up in distilled water. Clonazepam and the internal standard, methylclonazepam, were obtained from Hoffmann-La Roche (Nutley, NJ, U.S.A.). Clonazepam (10 mg) and 10 mg of methylclonazepam (internal standard) were

dissolved in 100 ml of methanol. These standards were stable at 4°C for at least six months. The working solution was prepared by diluting the stock solution 100-fold with water to obtain a solution containing 1 μg/ml of each drug. The serum standards containing 10, 25, 50, 100 ng/ml clonazepam were prepared by adding working solution to pooled drug-free human serum. Serum and plasma were used interchangeably. The serum standards are stable at 4°C for at least two weeks. The working internal standard was made by combining equal volumes of internal standard solution and glycine buffer to obtain a solution having a concentration of 50 ng/ml. The working internal standard is stable for one week at 4°C.

Buffers. Phosphate buffer (0.02 *M*) was prepared by dissolving 2.7 g of anhydrous potassium phosphate monobasic crystals in 1 l of distilled water. The pH of this solution was adjusted to 3.8 with orthophosphoric acid.

Glycine buffer (1 *M*) was made by dissolving 75.1 g of glycine (amino acetic acid, Sigma, St. Louis, MO, U.S.A.) in 1 l of water, and adjusting the pH to 10.5 with sodium hydroxide.

Mobile phase

The acetonitrile—phosphate buffer (30:70) mobile phase was prepared by adding 300 ml of acetonitrile to 700 ml of 0.02 mol/l phosphate buffer (pH 3.8). This solution was filtered before use.

Procedure

Place Bond-Elut columns on the top of the Vac Elut vacuum manifold. Pass two column volumes of methanol and distilled water through each column. Disconnect the vacuum as soon as the water has run through the columns to prevent them from drying out. Place 100 μl of internal standard (methylclonazepam) in 1 *M* glycine buffer onto each column, then pipette 1 ml of standard, control or patient sample onto each column. Connect the vacuum and wash each column with two column volumes of distilled water followed by 50 μl of methanol. Disconnect the vacuum, and place a rack containing appropriately labeled 75 × 10 mm glass tubes to collect eluent. Add 200 μl methanol to each column, and connect the vacuum and collect eluent. Add another 200 μl of methanol, collect, and combine the eluents.

Evaporate the methanol to dryness under a gentle stream of air in a water bath at 45°C. Reconstitute with 40 μl of methanol and inject all of the sample into the high-performance liquid chromatograph.

RESULTS

Recovery

Analytical recovery was calculated on drug-free pooled human serum spiked with known amounts of clonazepam to achieve the concentrations shown in Table I. A constant amount of internal standard was added to each sample. The samples were then processed as described and the recoveries shown in Table I were obtained. The analytical recovery ranged from 91% to 99% over the entire range. Absolute recovery of the drug averaged about 50%.

TABLE I

RECOVERY OF CLONAZEPAM FROM PLASMA SAMPLES ($n = 20$)

Drug added (ng/ml)	Drug recovered (ng/ml)	Standard deviation (ng/ml)	Coefficient of variation (%)	Percentage recovery
10	9.30	0.37	4	93
25	22.60	1.40	6	91
50	47.00	1.25	3	94
100	99.90	0.92	1	99

Linearity

Clonazepam was added to plasma in amounts equivalent to 15—100 μg/l, and a constant amount of internal standard was added to each sample. Concentration and peak height ratios were linear over this range.

Detection and sensitivity

The drugs and internal standard were detected at 254 nm, 1 ng of clonazepam standard could be detected by monitoring at 0.008 a.u.f.s. The minimum detectable concentration was 2 ng when 1 ml of serum sample was extracted. The sensitivity of this method allows for easy quantitation of 5 ng/ml clonazepam in 0.5 ml of serum.

Precision

Precision data were obtained by analyzing plasma samples containing two different concentrations of the drug as shown in Table II. For within-run determination the coefficients of variation (C.V.) ranged from 1.6% to 5%; for day-to-day precision, the C.V. ranged from 2.6% to 11%.

TABLE II

PRECISION OF CLONAZAPAM ASSAY

Concentration (ng/ml)	S.D. (ng/ml)	Coefficient of variation (%)
Within-run ($n = 14$)		
13.60	0.70	5.0
45.40	0.70	1.6
Day-to-day ($n = 10$)		
13.70	1.50	11.0
45.20	1.20	2.6

Interference

Possible interference from commonly used drugs was assessed by injecting concentrations of drugs that might be expected in overdose situations and measuring their retention times (Table III). Any drug found to elute sufficiently close to clonazepam or the internal standard was further evaluated by adding known amounts of the interfering drug to drug-free pooled plasma so as to quantitate the effect on clonazepam or the internal standard.

Carbamazepine, which normally interferes in the normal-phase assay of clonazepam, posed no problem. Furthermore, chlordiazepoxide could be resolved from clonazepam with the 25-cm column, a separation that was not possible in the other methods. Thus, none of the drugs tested were found to interfere.

Accuracy

Fig. 1 illustrates typical chromatograms obtained by this procedure: seventeen samples were assayed for clonazepam using the LC method. The results were compared with those obtained by GLC [9]. The coefficient of correlation was 0.973, the slope 0.744 and the intercept 6.2.

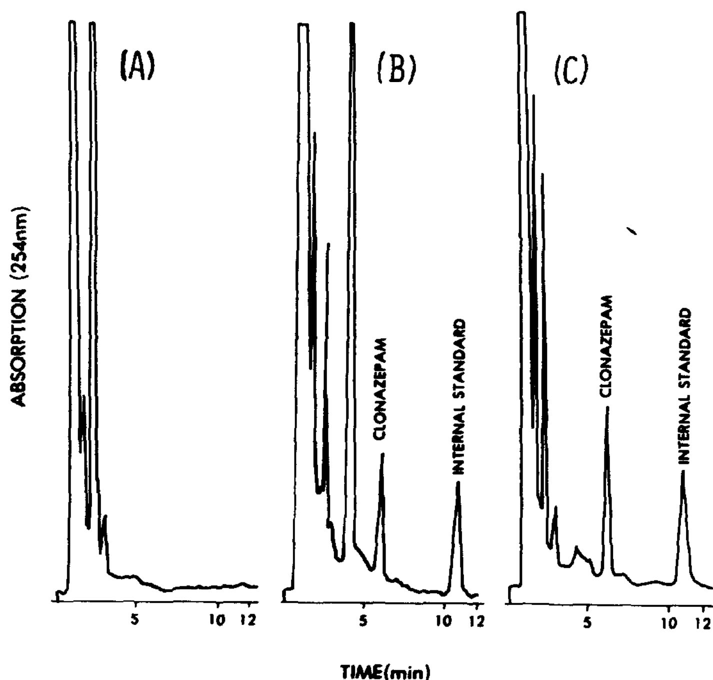

Fig. 1. Chromatogram of (A) clonazepam-free serum; (B) a patient's serum with 42 μg/l clonazepam; (C) a patient's serum with 57 μg/l clonazepam.

DISCUSSION

Clonazepam is an established anticonvulsant agent widely used in the treatment of epilepsy [25, 26]. The narrow therapeutic index and risk of increased seizure frequency in cases of overdose make routine monitoring of clonazepam plasma concentrations during treatment with the drug not only useful but necessary [26]. The high-performance liquid chromatographic procedure described would be adequate for such a purpose.

In the development of our present assay method, various chromatographic conditions were evaluated by injecting a solution of the drug in methanol to assess the effect of different parameters such as the pH of the mobile phase, the composition of the mobile phase, and the detection wavelength. All these factors were adjusted until optimal assay conditions were achieved.

TABLE III

RETENTION TIMES OF SOME OTHER DRUGS WITH THE MOBILE PHASE

Drug	Retention time (min)	Drug	Retention time (min)
Allyl cyclopentenyl barbituric acid	1.9	Mephobarbital	3.8
Alphenol	1.9	Meprobamate	N.D.
Amitriptyline	27.0	Mesoridazine besylate	11.2
Amobarbital	3.4	Methadone	N.D.
Anafranil	N.D.*	Methaqualone	6.4
Aprobarbital	1.2	Methyl clonazepam	11.0
Barbituric acid	0.4	Methyl nitrazepam	8.6
Butabital	1.5	Methyprylon	1.4
Butalbital	2.2	N-Acetyl procainamide	N.D.
Carbamazepine	3.8	Pentathal	7.6
Chlordiazepoxide	7.0	Nirvanol	1.0
Chlorpromazine	42.0	Nortriptyline	21.0
Clonazepam	6.0	Oxazepam	17.0
Demoxepam	3.0	Pentobarbital	3.2
Desipramine	16.0	Perphenazine	40.2
Diallyl barbituric acid	1.0	Phenobarbital	1.4
Diazepam	16.8	Phenytoin	3.2
Ethinamate	N.D.	Procainamide	N.D.
Ethosuximide	0.6	Promazine	21.0
Flurazepam	10.0	Propoxyphene	N.D.
Gentamicin	N.D.	Propranolol	5.0
Glutethimide	4.4	Protriptyline	16.0
Heptobarbital	3.2	Quinidine	2.3
Hexobarbital	3.4	Secobarbital	4.4
Imipramine	22.0	Thioamyl	1.1
Lidocaine	1.6	Thioridazine hydrochloride	N.D.
Mebutamate	N.D.	Trifluoperazine hydrochloride	N.D.
Medazepam	25.0	Triflupromazine hydrochloride	N.D.
Meperidine	2.8	Tybamate	N.D.
		Vinbarbital	1.9

*N.D. = Not detected.

At 306 nm it was possible to monitor the concentration of clonazepam, but the peak height of clonazepam was 20% greater at 254 nm.

In several published methods phosphate buffer or borate butter was employed for the extraction of clonazepam, but we found that the glycine buffer (1 mol/l) used by Wad and Hanifl [2] for the TLC determination of diazepam and its metabolites to yield better recovery of clonazepam from serum than phosphate buffer.

Different compositions of the mobile phase (phosphate buffer—acetonitrile) were investigated, for example 60:40, 65:35, 72:28 and 70:30. The 70:30 composition was found to be the best. With the other combinations one of two things usually happened. Either the drug eluted too fast and was interfered with by serum constituents, or the internal standard peak was not sharp. Furthermore, with a flow-rate of 2 ml/min and 70:30 composition of mobile phase the assay could be performed in just 12 min.

Methylclonazepam was chosen as an internal standard because it is chemically similar to clonazepam. The usefulness of this internal standard can be appreciated by considering the fact that of over seventy drugs tested for interference none was found to interfere with clonazepam and the internal standard.

The time factor should also be considered. As the chromatography is complete in 12 min, a skilled technician can analyze thirty samples in about 6 h. This is possible by the solid-phase extraction method which can process ten samples in 15 min.

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