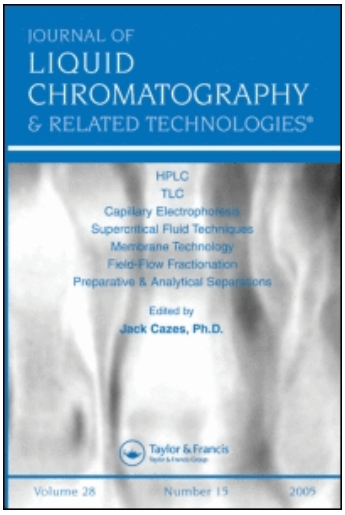


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High-Performance Liquid Chromatographic Determination in Human Plasma of a Anticonvulsant Benzodiazepine: Clonazepam

S. Bouquet[a]; P. Aucouturier[a]; A. M. Brisson[a]; Ph. Courtois[a]; J. B. Fourtillan[a]
[a] Département de Pharmacocinétique Centre Hospitalier, Universitaire JEAN-BERNARD, POITIERS-CEDEX, FRANCE

To cite this Article Bouquet, S. , Aucouturier, P. , Brisson, A. M. , Courtois, Ph. and Fourtillan, J. B.(1983) 'High-Performance Liquid Chromatographic Determination in Human Plasma of a Anticonvulsant Benzodiazepine: Clonazepam', Journal of Liquid Chromatography & Related Technologies, 6: 2, 301 — 310
To link to this Article: DOI: 10.1080/01483918308066891
URL: http://dx.doi.org/10.1080/01483918308066891

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 301-310 (1983)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION IN HUMAN PLASMA OF A ANTICONVULSANT BENZODIAZEPINE : CLONAZEPAM

S. BOUQUET, P. AUCOUTURIER, A.M. BRISSON, Ph. COURTOIS and J.B. FOURTILLAN
Département de Pharmacocinétique
Centre Hospitalier Universitaire JEAN-BERNARD
86021 POITIERS - CEDEX - FRANCE

ABSTRACT

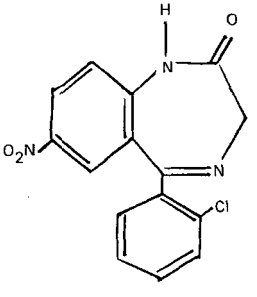
A rapid and specific high-pressure liquid chromatography method for determination of clonazepam in human plasma is described.

The analysis is linear for concentrations ranging from 5 to 100 ng. ml^{-1} plasma for clonazepam.

The method is applicable to quantitation of clonazepam in human plasma of subjects receiving 0.05 at 0.20 $mg.kg^{-1}$ orally, with satisfactory accuracy and precision.

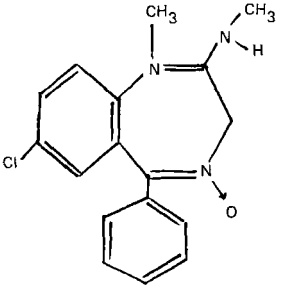
INTRODUCTION

In recent years, the use of clonazepam (5 ortho-chlorophenyl, 7 nitro, 2,3-dihydro, 1-4 benzodiazepine 2-one) has been developed as a treatment for convulsions (8 , 12).



CLONAZEPAM :

(5-ortho-chlorophenyl 7-nitro 2,3-dihydro 1,4-benzodiazepine 2-one)



CHLORDIAZEPOXIDE : (Internal standard)

(7-chloro-2 methylamino-5 phenyl-3H-1,4 benzodiazepine-4-oxide).

0148-3919/83/0602-0301$3.50/0

Recently, a few authors have advised the prescription of anticonvulsant drug monotherapy (2, 10, 12).

Meanwhile, it is difficult to understand why an identical drug dosage may exert a toxic effect in one patient and a therapeutic, or no reponse in another patient. Numerous clinical studies (2, 8, 9, 13) have adequately demonstrated the importance of total plasma concentrations of clonazepam in relation to its efficacy. Several analytical methods have been proposed for benzodiazepine determination in biological fluids : gas liquid chromatography (GLC) (4, 5) and high performance liquid chromatography (HPLC) (1, 3, 11, 13).

The GLC methods require somewhat lengthy clean-up procedures and, in some cases, derivatization or acid hydrolysis to the more volatile benzophenones (4, 5, 7).

High-performance liquid chromatography (HPLC) involves relatively simple extraction, no derivatization and U.V. detection to give high sensitivity, good stability, and linearity over wide concentration ranges.

This paper demonstrates the use of HPLC in a reverse phase mode to separate clonazepam from endogenous compounds, in human plasma samples.

An internal standardization technique is employed, using a structurally related benzodiazepine (chlordiazepoxide) as the internal standard.

METHODS

The procedure involves the addition of chlordiazepoxide as the internal standard. After addition of 200 µl 0.5 N NaOH ; samples are extracted using ether.

After evaporation of the organic solvant, the residue is dissolved in mobile phase and the benzodiazepine is analysed isocratically by reverse phase high-pressure liquid chromatography with 40 % (v/v) acetonitrile in distilled water as eluant. The effluent is monitored by U.V. detection at 254 nm.

Apparatus

The chromatographic determinations were performed with a Waters Associates Liquid Chromatograph Model No 440-03773, equipped with a model 440 absorbance detector (254 nm wavelength), a U_6K injector, a flow pump Model 6000 A (Waters Associates Inc. Milford, Mass. 01757, and a 10 mV recorder Omniscribe (Houston Instruments, Gistel, Belgium).An octadecylsilane µ-Bondapack C_{18} column 3.9 mm i.d.X 30 cm long (Waters Associates) was used under ambient conditions, for the separation. The isocratic mobile phase consisted of acetonitrile/bi-distilled water (40/60, v/v). The solution was filtered through 0.22 µm pore membrane filter type GS-ester of celluose (Millipore Corp., Bedford, Mass. 01730) and the flow rate was 1.5 $ml.mn^{-1}$. Under these conditions, clonazepam and the internal standard (chlordiazepoxide) were eluted with retention times of 8.3 and 9.2 min., respectively, as illustrated in Figure 1.

Reagents and drugs :

Clonazepam and chlordiazepoxide, pharmaceutical grade, were obtained from Roche Laboratories (Neuilly - France). The water was double-distilled and filtered through a 0,22 µ (type GS - ester of cellulose) filter (Millipore, Corp., Bedford, Mass. 01730).

Methanolic stock solutions of clonazepam and chlordiazepoxide (the internal standard) were prepared at a concentration of 100 µg/ml and could be stored at 4°C during a week, in the dark. For the determination of low concentrations, extraction solvent : diethyl ether, n-hexane and acetonitrile of high purity (Chrom AR Nanograde, Byk-Mallinckrodt, Wessel, GFR) were used.

U.V. grade methanol and sodium hydroxyde 30 % type RP, were purchased from (Prolabo, Paris, France) and (Carlo Erba, Milan, Italy), respectively.

Extraction procedure :

Into a screw-stoppered test tube, put one ml of plasma ; add 50 µl of aqueous chlordiazepoxide solution (10 $\mu g.ml^{-1}$), adjust to pH 9.5 with 0.5 N sodium hydroxyde (about 0,200 ml for 1 ml plasma), homogenize by slow rotation.

The drug was extracted with 5 ml of ethyl-ether by shaking mecanically for 10 min and centrifuged for 5 mn at 3000 rpm. An aliquot of the upper organic layer was transferred to another test-tube.

Re-extract the sample, proceeding as before. Combine the ethereal extracts and evaporate to dryness under dry nitrogen at 35°C.

Take up the residue with 200 µl of 0.2 M HCl, add 200 µl of n-hexane, homogenize for 30 sec. on a vortex mixer and centrifuge at 3000 rpm for 3 min. Remove and discard the upper hexanic phase (containing the lipids extracted from the plasma). An aliquot of 100 µl aqueous phase was injected into the HPLC system

RESULTS AND DISCUSSION

We found a linear correlation between the concentration of clonazepam and the ratio of peak heights : clonazepam : internal standard, in the range between 12.5 and 100 $ng.ml^{-1}$ of the plasma samples. (Figure 1 and 1 Bis) R = 0.99. Addition of an internal standard to the plasma prior to extraction allows quantitative measurements.

No interfering peak with the retention time of Clonazepam was present in extracts of "blank" control plasma (Figure 2).

For lower concentrations, it is advisable to start with 2 ml of plasma and work at 0.005 A.U.F.S. sensitivity. Under these conditions, the limit of detection of clonazepam was approximately 10 $ng.ml^{-1}$ of plasma.

Figure 3 illustrates the chromatographic profile of a human plasma extract, from a patient receiving daily oral administration 4 mg clonazepam. The known metabolites of clonazepam in which amino and acetamido substituents

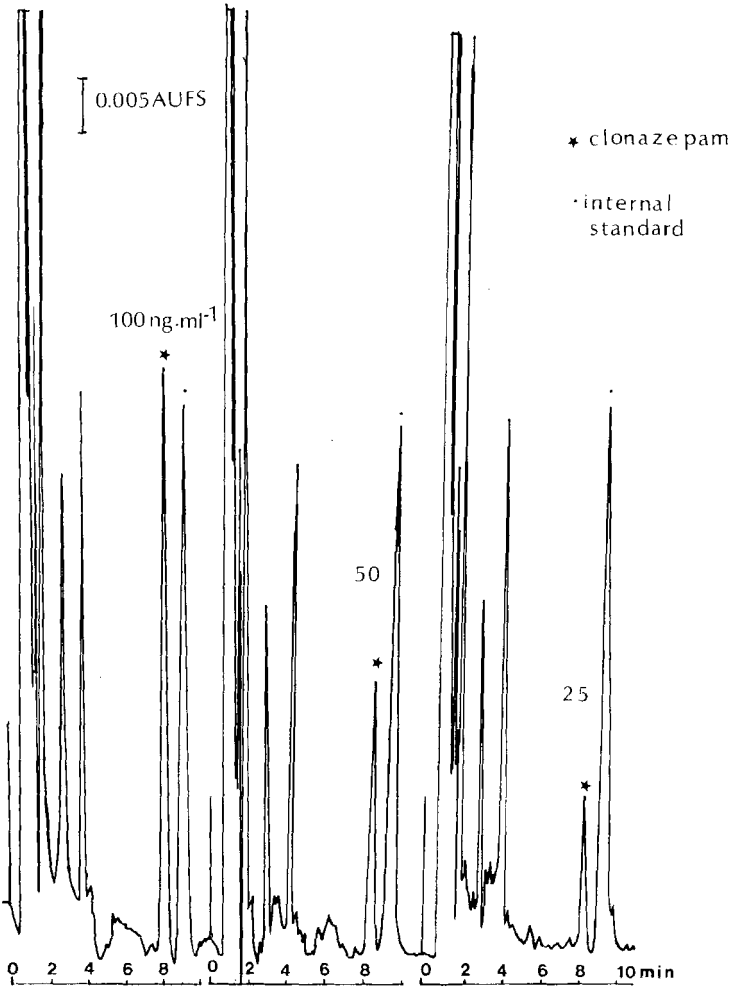


FIGURE 1 : CHROMATOGRAM OF CLONAZEPAM

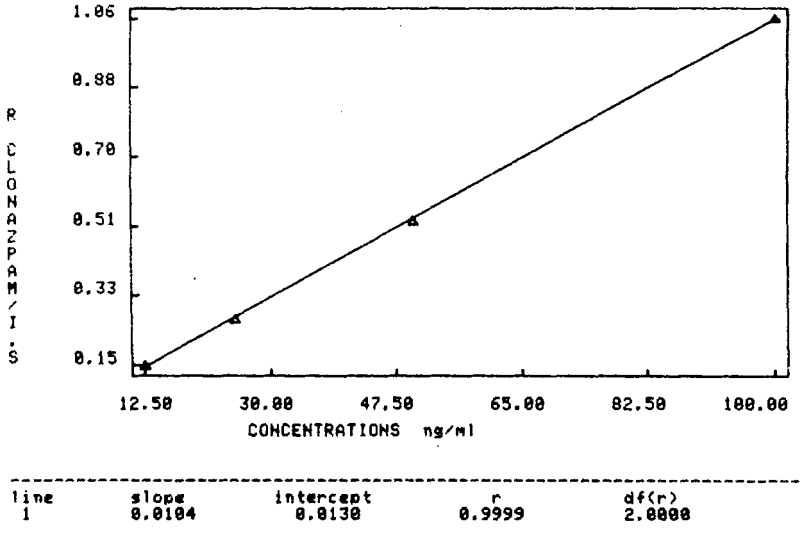


FIGURE 1 BIS : REGRESSION ANALYSIS CLONAZEPAM

are present on the drug molecule, should not interfere with the analysis of clonazepam (Figure 3).

Figure 4 : the separation of a test mixture of benzodiazepines (2, 3, 4, 5, 6, 7) and carbamazepine (1), showing the good selectively of the phase system for clonazepam (3).

In this work, flunitrazepam (5) and desmethyldiazepam (7) were found interfere with chlordiazepoxide (4) and clobazam (6), respectively.

Nevertheless, the peaks given by oxazepam (2) and carbamazepine (1) were not completely separated.

Reproducibility of the extraction procedure was determined by extracting a plasma sample containing 50 $ng.ml^{-1}$ clonazepam daily over a 10 day period, with the following results (n = 10, CV = 3.6 %).

Another standardization sample containing 200 $ng.ml^{-1}$ in water was directly injected daily, producting the following values (n = 10, CV = 4.5 %).

Steady-state plasma values for individual clonazepam are published (4, 6), obtained by other techniques, from adult chronic patients undergoing continuous treatment (4 to 8 mg daily). We chose values similar to these (I.e 20 to 60 $ng.ml^{-1}$). No appreciable loss in resolution was observed during the chromatography of more than 200 plasma sample extracts.

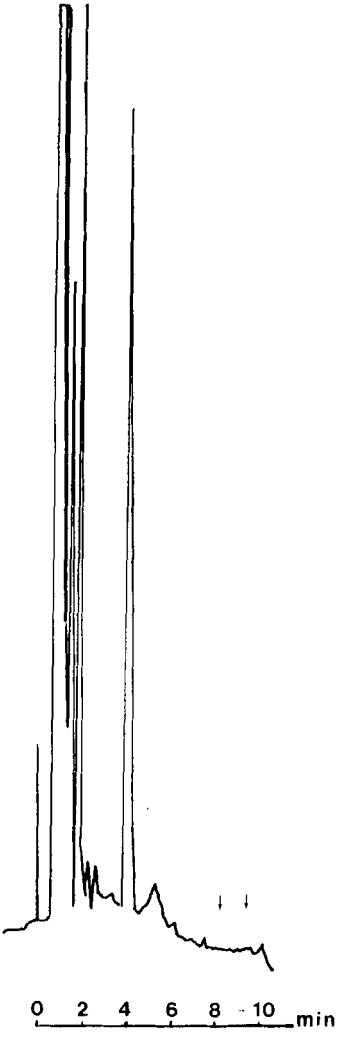


FIGURE 2 : A BLANK PLASMA EXTRACT

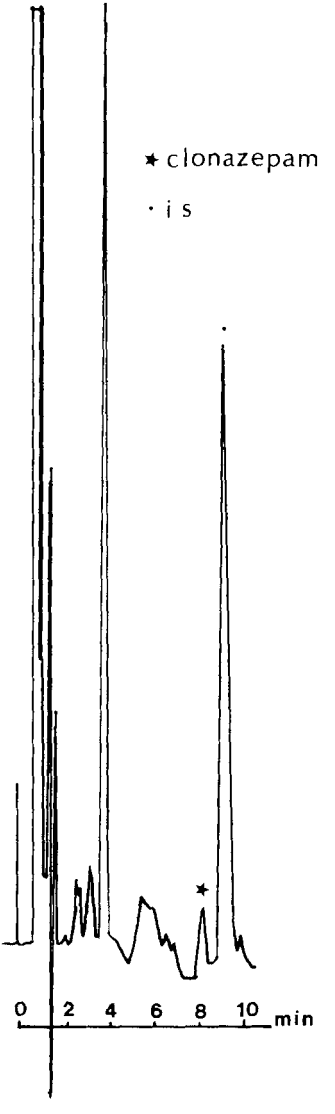


FIGURE 3 : CHROMATOGRAM OF A HUMAN PLASMA

(★ 21 $ng.ml^{-1}$)

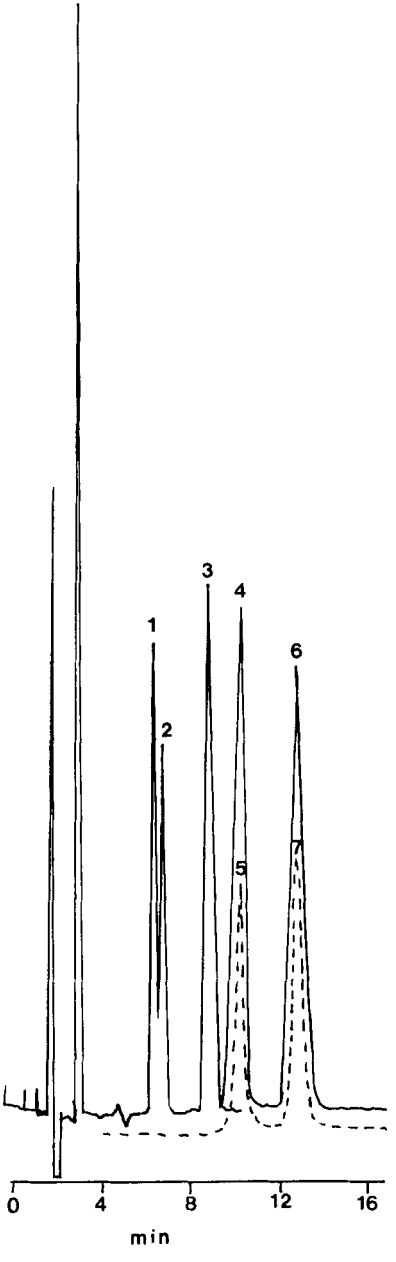


FIGURE 4

In conclusion, we consider that reverse-phase HPLC is the most suitable method of analysis for determining clonazepam in human plasma because of its specificity, sensitivity, simplicity and speed of execution.

REFERENCES

1. R.F. Adams, F.L. Vandemark, "Simultaneous high pressure liquid chromatographic determination of some anticonvulsants in serum", Clin. Chem., 22, 1, 25 (1976)

2. A. Baruzzi, G.P. Cabrini, V.A. Gernam-Sironi and P.L. Morselli, "Anticonvulsant plasma levels monitoring in epileptic patients undergoing stereo EEG in Gardner Thorpe (eds),"Clinical Pharmacology of Antiepileptic Drugs", vol. II, Pitman Medical, publisher Kent, (1977), p. 197.

3. R.R. Brodie, L.F. Chasseaud and T. Taylor, "High-performance liquid chromatographic determination of benzodiazepines in human plasma", J. Chrom., 150 : 361, (1978).

4. J.P. Cano, J. Guintrand, C. Aubert, A. Viala, "Determination of flunitrazepam, desmesthylflunitrazepam and clonazepam in plasma by gas liquid chromatography with an internal standard", Arzneimittel Forsch, 27 : 2, 338, (1977).

5. J.A. De Silva, I. Berkersky, C.V. Puglisi, M. Brooks, R.E. Weinfeld, "Determination of 1,4-benzodiazepines and diazepin-2-ones in blood by electron capture gas-liquid chromatography", Anal. Chem., 48 : 1, 10, (1976).

6. E. Eschenhof, "The pharmacokinetics of clonazepam", Arzneimittel Forsch, 23 : 390, (1973).

7. A. Frigerio, K.M. Baker and G. Belvedere, "Gas chromatographic degradation of several drugs and their metabolites", Anal. Chem., 45 : 1846, (1973).

8. J. W. Lance, M. Antony, "Sodium valproate and clonazepam in the treatment of intractable epilepsy", Arch. Neurol., 34 : 14, (1977).

9. P.L. Morselli, "Clinical significance of monitoring plasma levels of benzodiazepine tranquilisers and antiepileptic drugs", Neuro-Psychopharmacology, edited by P. Deniker, C. Radouco-Thomas, A. Villeneuve, Pergamon Press Oxford & New York (1978), p. 877.

10. E.H. Reynolds, S.D. Shorvon S.D., "Medical treatment of epilepsy : monotherapy versus polytherapy, New Press Med., 10 : 3717, (1981).

FIGURE 4 : SEPARATION OF A TEST MIXTURE OF DIFFERENT BENZODIAZEPINES AND CARBAMAZEPINE.
COLUMN : μ BONDAPACK C_{18} 3.9 MM I.D. x 30 cm L
ELUENT : ACETONITRILE - BI-DISTILLED WATER (40 : 60 v/v)
FLOW RATE : 1.5 $ml.mm^{-1}$.
PEAKS : 1 = CARBAMAZEPINE , 2 = OXAZEPAM, 3 = CLONAZEPAM,
4 = CHLORDIAZEPOXIDE, 5 = FLUNITRAZEPAM,
6 = CLOBAZAM, 7 = DESMETHYL-DIAZEPAM.

11. G.C. Skeller, J. Meier, B.I. Knight., B. Whiting, "The application of HPLC to the determination of some 1-4 benzodiazepines and their metabolites in plasma", Brit. Clin. Pharmacol., 5 : 483, (1978).

12. E.A. SWINYARD and A.W. Castellion, "Anticonvulsant properties of some benzodiazepines", J. Pharmacol. Exp. Ther., 151 : 369, (1966).

13. U.R. TJADEN, M.T.H.A. Meeles, C.P. Thys and M. Vanderkaay, "Determination of some benzodiazepines and metabolites in serum, urine and saliva by high-performance liquid chromatography", J. Chrom., 181 : 227, (1980).