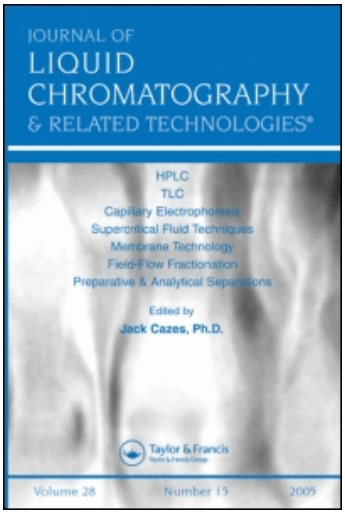

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A High-Performance Liquid Chromatographic Method for the Determination of Doxefazepam in Human Plasma Using a Solid-Phase Extraction Column

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A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF DOXEFAZEPAM IN HUMAN PLASMA USING A SOLID-PHASE EXTRACTION COLUMN

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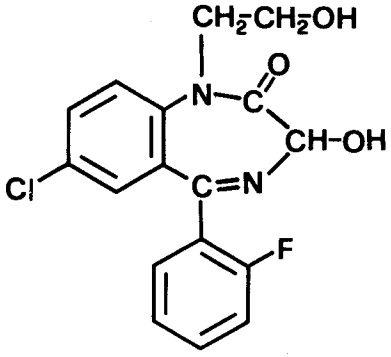
ABSTRACT

A procedure for the rapid, quantitative isolation of doxefazepam from plasma with Supelclean LC-18 cartridges is described together with a sensitive HPLC assay for the quantitative determination of the drug.The recovery of doxefazepam was greater than 80 % over an investigated range of 0.1-2.0 μg/ml of plasma. The column extraction of doxefazepam coupled with the versatility of HPLC make this procedure well suited for detailed pharmacokinetic studies and as well as routine plasma analysis of doxefazepam.

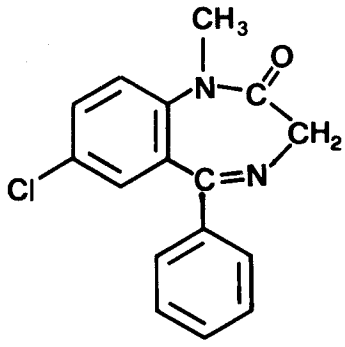
INTRODUCTION

Doxefazepam, 1(2-hydroxyethyl) -3-hydroxy -7-chloro-1,3- dihydro -5-(o-fluorophenyl)-2H-1,4 -benzodiazepine -2-one, is a member of the 1,4-benzodiazepine class of compounds and was synthesized by Tamagnone et al.(1); it shows hypnotic effects in man (2,3). Many reports have been published on the determination of doxefazepam in biological fluids, gas chromatography (GC) being used extensively (4,5). The GC methods require somewhat lengthy clean-up procedures, and in some cases derivatization. Only one report has appeared on the analysis of doxefazepam by high-performance liquid chromatography (HPLC) (6); in that report, however, doxefazepam was extracted using the liquid-liquid technique.

The present paper reports the successful isolation of doxefazepam in plasma by the chromatographic method using Supelclean C-18 cartridges. Diazepam, structurally related to doxefazepam, is chosen as internal standard (Fig.1).



DOXEFAZEPAM



DIAZEPAM

FIGURE 1. Structural formulae of doxefazepam and of the internal standard diazepam.

EXPERIMENTAL

Reagents and Materials

Doxefazepam was purchased from Schiapparelli (Torino, Italy). Diazepam was obtained from the Department of Pharmacology of this University. Water (HPLC grade) was obtained from double distillation in glass and purification through a "Milli-Q-Water purification System" (Millipore, Bedford, MA, U.S.A.). Methanol (HPLC grade) was purchased from Farmitalia Carlo Erba (Milano, Italy). Supelclean solid phase extraction tubes

(SPE) and a vacuum manifold were obtained from Supelco (Bellafonte, PA, U.S.A.).

Chromatography

The chromatographic system consisted of a U6K injector and a M6000A pump connected to a 740 Data Module integrator with a model 440 UV detector operating at a wavelength of 280 nm and a sensitivity of 0.01 absorbance units, full scale (Waters, Associates, Inc., Milford, MA, U.S.A.). Separation was performed at room temperature on a μ-Bondapak C-18 reversed-phase column (0.4 by 30 cm, Waters) with a Supelguard LC-18 precolumn (0.4 by 2 cm, Supelco). The mobile phase was a mixture methanol-water (20:80, v/v) of. The flow-rate was 2.0 ml/min.

Extraction procedure

The disposable extraction columns were placed on the top of the SPE vacuum manifold, and 1 ml of methanol followed by 2 ml of water were used to desorb any organic impurities; thus the silica

packing was wet prior to the introduction of plasma sample. 1.0 ml of plasma was transfered into each column, containing 100 µl of a methanolic solution (2 µg/ml) of internal standard; the vacuum was then connected and each column was washed with two column volumes of distilled water followed by 100 µl of methanol. The vacuum was disconnected, and the rack containing appropiate glass tubes was set in place to collect the eluate. Doxefazepam was eluted from the columns with 400 µl of methanol. The eluates were evaporated to dryness under a gentle stream of nitrogen. The dried extracts were redissolved in 200 µl of the mobile phase. 25 µl of each sample were injected into the chromatograph.

RESULTS AND DISCUSSION

A HPLC procedure for the determination of doxefazepam in human plasma has been developed. The recovered residue was found to be essentially free of sample matrix and showed low background. The chromatogram obtained from a plasma sample is

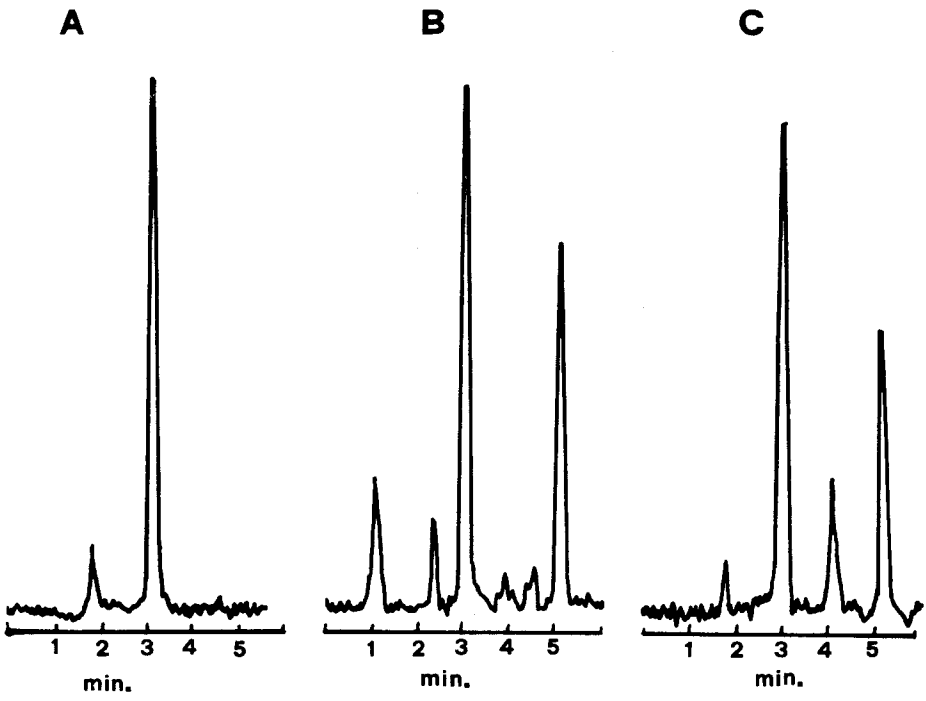


FIGURE 2. (A) Drug-free plasma containing the internal standard; (B) drug-free plasma reconstituted with 1.0 μg/ml doxefazepam and the internal standard; and (C) a patient sample wich was taken 2 h after an oral dose of 20 mg doxefazepam. Retention times: internal standard 3.1 min.; doxefazepam 5.2 min..

given in Fig.2. The peaks corresponding to the internal standard and doxefazepam were well resolved under the conditions described above and no endogenous compound extracted at the same time interfered with them. Retention times for

diazepam and doxefazepam were 3.10 and 5.20 minutes, respectively. The analytical recovery of doxefazepam from plasma was determined by comparing the peak area ratios (doxefazepam to internal standard) obtained by analyzing extracted, spiked plasma specimens, to the peak area ratios obtained by direct injection of methanolic solutions of doxefazepam and internal standard containing amounts of the two drugs equal to those in the plasma samples. The results are given in Table I. Calibration curves (peak area ratio versus concentration) were obtained by analyzing plasma standards containing doxefazepam in concentrations ranging from 0.1 to 2.0 μg/ml. The calibration curves showed linear responses with a correlation coefficient of 0.988 on ten data points. The sensitivity was determined by repeated analysis of spiked plasma samples containing 0.1 μg/ml of doxefazepam and 2 μg/ml of internal standard. The mean peak area ratio was found to be 0.008 (n = 5, C.V. = 9.3%) indicating that 0.1 μg/ml of doxefazepam in plasma can be determined with acceptable precision in 1.0 ml plasma samples. Within-run

TABLE I. RECOVERY STUDIES (n=5)

Amount of drugs and extract μg/ml	mean value of conc. found μg/ml	Percent recovery %	C.V.(%)
0.1	0.09 ± 0.008	81.6-98.4	9.3
0.5	0.48 ± 0.034	89.1-102.9	7.2
1.0	0.95 ± 0.046	90.4-99.6	4.6
1.5	1.47 ± 0.02	96.6-99.3	4.2
2.0	1.96 ± 0.070	94.5-101.5	3.6

TABLE II. PRECISION STUDIES

Doxefazepam	Mean ± SD μg/ml	C.V. %
	Within-run(n=7)	
	0.53 ± 0021	4.1
	1.46 ± 0.046	3.2
	2.05 ± 0.016	3.0
	Between-run(n=5)	
	0.42 ± 0.037	9.0
	0.89 ± 0.055	6.2
	1.10 ± 0.047	4.3
	1.40 ± 0.058	4.2

precision was calculated from the mean values for ten duplicate measurements. The mean concentration, standard deviation, and coefficient of variation for within-run and between-run variances are shown in Table II. The

day-to-day precision was C.V. 2.9-3.8%, calculated from data obtained on standard samples with various concentrations analyzed over one month. The solid-phase extraction procedure described in the present paper is simple,rapid, and does not require large amounts of organic solvents. Ten samples can be processed in about twelve minutes.

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