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Note

Determination of chlordiazepoxide and its metabolites in human plasma by reversed-phase high-performance liquid chromatography

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Many analytical methods of analysis have been developed in the past few years for determinating chlordiazepoxide and its metabolites in body fluids, using colorimetry [1], spectrophotofluorimetry [2,3], gas chromatography [4,5], differential pulse polarography [6], radioimmunoassay [7] and thin-layer chromatography with densitometry [8]. Each method has its limitations, permitting the determination either of chlordiazepoxide only and not of its metabolites or of only some of these.

With the advent of high-performance liquid chromatography (HPLC) and its rapid acceptance in the biomedical field, it is possible to solve the problem speedily, as has been predicted [5,9]. Recently two papers have appeared dealing with the determination of chlordiazepoxide and its metabolites in plasma or serum by means of reversed-phase HPLC [10,11]. The first [10] disregards two active metabolites of chlordiazepoxide, demoxepam and desmethyldiazepam, and uses the gradient technique, in our view unjustified. The second [11], the starting point for the method we have developed, uses a type of extraction that is unsuitable for demoxepam because of the low recovery (3.8%). Further this method of extraction does not allow for purification of the body fluids from their lipids, which damage the columns and reduce the reproducibility of the results. At least, the particle size of the chromatographic column support (5 μm) is somewhat critical and, in our view, not recommendable for routine work.

Recently, a basic paper [12] dealing with HPLC of Librium® and metabolites in human plasma, was published; this paper differs from ours, above all, in chromatographic conditions. In fact, both analytical methods rely on a double property common to many benzodiazepines to be chromatographed (unionized molecules) either in acid [12] or in alkaline medium (our paper).

We describe here a new method of determining chlordiazepoxide and its active metabolites in human plasma, that may be used for studying pharmacokinetics in man during long-term chlordiazepoxide therapy [13].

EXPERIMENTAL

Chromatographic system

The determinations were carried out on a chromatographic system made up as follows: Altex Model 110 A pump (Altex Scientific, Berkeley, Calif., U.S.A.), UV—visible (200—850 nm) Kontron-Uvicon 725 spectrophotometric detector (Kontron, Zurich, Switzerland) operating at a wavelength of 260 nm with 0.02 a.u.f.s., Rheodyne Model 712 injector with a 20-μl loop (Rheodyne, Berkeley, Calif., U.S.A.), an Hibar chromatographic column filled with LiChrosorb RP-18 (10 μm) (E. Merck, Darmstadt, G.F.R.) 250 mm × 4 mm I.D., and/or an Altex column filled with LiChrosorb RP-18 (10 μm), 250 mm × 3.2 mm I.D., and a precolumn filled with Corasil™ (37—50 μm) (Waters Assoc., Milford, Mass., U.S.A.). The detector was coupled through an interface to a Sigma 10 Data System chromatographic computer (Perkin Elmer, Norfolk, Conn., U.S.A.) and the calculations were made according to an internal standard method.

The eluent was a mixture of acetonitrile—0.1% ammonium carbonate (31:69), in isocratic conditions with a flow-rate of 2 ml/min (ca. 1300 p.s.i.).

In the above conditions the retention times of the substances of interest were: demoxepam ca. 4 min, desmethylchlordiazepoxide 5 min, nitrazepam (internal standard) 6 min 20 sec, chlordiazepoxide 7 min 45 sec, and desmethyldiazepam 11 min (Hibar column); demoxepam 2 min 20 sec, desmethylchlordiazepoxide 3 min, nitrazepam 3 min 40 sec, chlordiazepoxide 4 min 40 sec, desmethyldiazepam 6 min 30 sec (Altex column).

Reagents and drugs

Acetonitrile and *n*-hexane Lichrosolv were from E. Merck and ammonium carbonate, sodium hydroxide type RPE and diethyl ether for pesticides from C. Erba (Milan, Italy). The water was double-distilled and filtered through a 2-μm filter, Chlordiazepoxide, N-desmethylchlordiazepoxide, demoxepam, N-desmethyldiazepam and nitrazepam, pharmaceutical grade, came from Hoffmann-La Roche (Basle, Switzerland).

Extraction of chlordiazepoxide and metabolites from human plasma

Into a screw-stoppered test tube (Sovirel 30, Paris, France) put 1 or 2 ml human plasma (from a pool of plasma from healthy subjects not treated with drugs for at least two weeks), adjust to pH 9 with 0.1 *N* sodium hydroxide (about 0.275 ml for 2 ml plasma), add 5 ml double-distilled water and homogenize by slow rotation. To the aqueous phase add 7 ml diethyl ether and extract mechanically for 10 min (Model K 30/300 three-dimensional agitator, Bicasa, Milan, Italy).

Centrifuge at 2500 *g* for ca. 3 min and carefully remove the ethereal phase. Re-extract the sample, proceeding as before. Combine the ethereal extracts and evaporate on a thermostatically controlled waterbath at 40 ± 1° in a light stream of pure nitrogen. Take up the residue with 100 μl of eluent mixture (for chromatography) containing 10 μg nitrazepam (internal standard), add 100 μl *n*-hexane, homogenize for 30 sec on a vortex mixer and then centrifuge at 1065 *g* for 2 min. Remove and discard the upper hexanic phase

(containing the lipids extracted from the plasma) with a 1-cm^3 syringe (special 2-in. 23 gauge needle, Becton-Dickinson, Toronto, Canada). Inject 20 μl into the chromatographic system, operating in the conditions described.

RESULTS AND DISCUSSION

We found a linear correlation between the concentration of the benzodiazepines and the ratio of the areas of the peaks, benzodiazepine/internal standard, in the range between 30 ng/ml and 3 μg/ml of the original plasma samples. For desmethyldiazepam the linearity range was between 50 ng/ml and 3 μg/ml. For lower concentrations it is advisable to start with 2 ml plasma and work at 0.01 a.u.f.s.; even at this sensitivity the instrumental noise is fairly low. The sensitivity limits for the drugs are the lower values of the linearity range (starting from 2 ml of human plasma samples).

This procedure is standardized for determinations on plasma samples from patients on long-term chlordiazepoxide therapy, but is also suitable for single 30-mg oral administration. Steady-state plasma values for individual benzodiazepines are published [6,14], obtained by other techniques, from patients undergoing chronic treatment (30 mg daily); we chose values similar to these

TABLE I

RECOVERY OF CHLORDIAZEPOXIDE AND ITS METABOLITES ADDED TO HUMAN PLASMA USING AN ALTEX COLUMN

Compound	Quantity added (μg/ml)	Recovery* (μg/ml)	Recovery** (%)
Chlordiazepoxide	1.20	1.190 ±0.028	99.0 ±2.3
	1.00	0.973 ±0.012	97.3 ±1.2
	0.60	0.599 ±0.007	99.9 ±1.19
	0.20	0.202 ±0.009	101 ±4.5
	0.10	0.103 ±0.0048	103 ±4.66
N-Desmethylchlordiazepoxide	0.40	0.3707±0.0068	92.7 ±1.84
	0.35	0.337 ±0.007	96.3 ±2.0
	0.30	0.271 ±0.005	90.3 ±1.85
	0.18	0.175 ±0.007	97.1 ±4.1
	0.12	0.106 ±0.0047	88.27±4.43
Demoxepam	0.45	0.341 ±0.0085	76 ±2.5
	0.30	0.220 ±0.0041	73 ±1.84
	0.20	0.153 ±0.004	76.4 ±2.6
	0.08	0.065 ±0.0018	81.6 ±2.83
	0.05	0.0422±0.002	84.5 ±4.72
N-Desmethyldiazepam	0.40	0.392 ±0.019	98 ±4.8
	0.30	0.2937±0.0093	98 ±3.2
	0.20	0.1935±0.0116	96.8 ±6

*Mean of 5 determinations, ± S.D. calculated on 5 plasma samples having the same nominal concentration.

**Mean recovery (%) ± S.D.

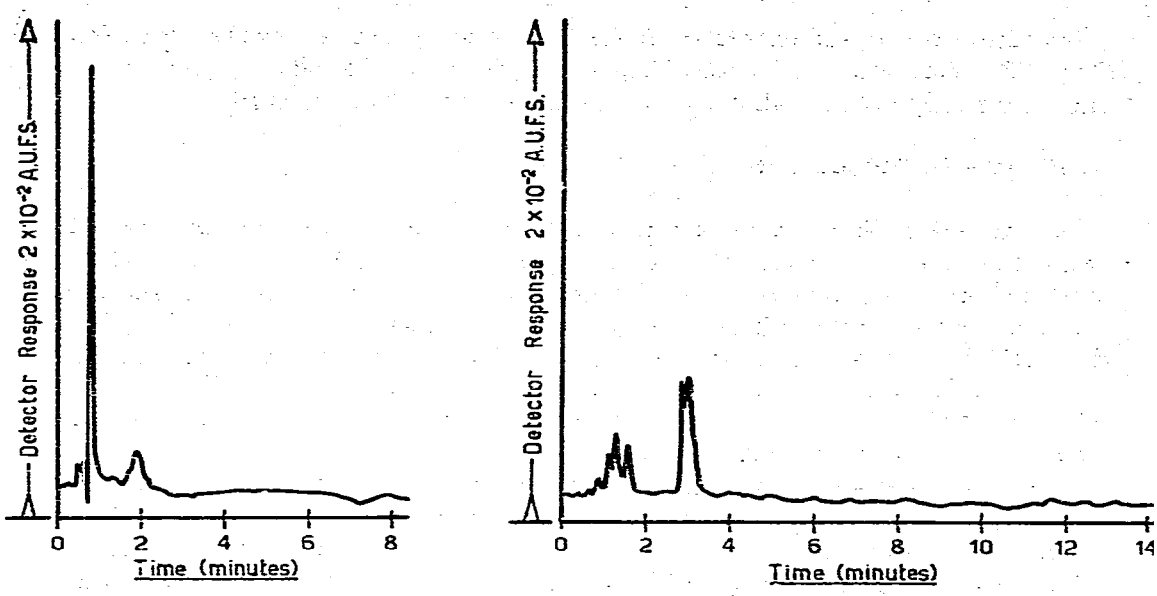

Fig. 1. Chromatogram of an extract of control human plasma using an Altex column.

Fig. 2. Chromatogram of an extract of control human plasma using an Hibar column.

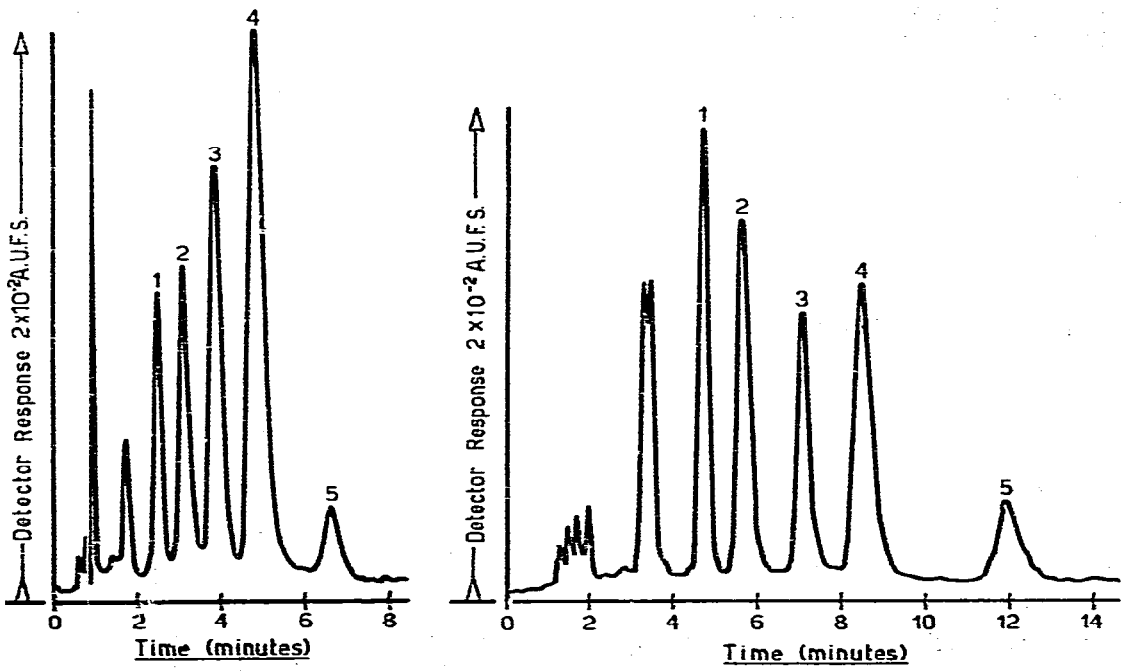

Fig. 3. Chromatogram of authentic standards recovered from control human plasma: 1, lemoxepam (0.4 μg/ml); 2, desmethylchlordiazepoxide (0.4 μg/ml); 3, nitrazepam; 4, chlordiazepoxide (1 μg/ml) and 5, desmethyldiazepam (0.4 μg/ml); column, Altex.

Fig. 4. Chromatogram of authentic standards recovered from control human plasma: 1, lemoxepam (0.45 μg/ml); 2, desmethylchlordiazepoxide (0.35 μg/ml); 3, nitrazepam; 4, chlordiazepoxide (0.48 μg/ml) and 5, desmethyldiazepam (0.4 μg/ml); column, Hibar.

for simulating plasma concentrations close to the real ones. When this method is employed to determine chlordiazepoxide and its metabolites after a single 30-mg dose of Librium it is necessary to bear in mind the fact that demoxepam and desmethyldiazepam are present at rather low concentrations about 30 h after administration [6], here the sensitivity can be increased by reducing the final volume of extract from 100 to 50 μl and/or using a larger loop. Table I gives the results. Also, some examples of chromatograms are given (Figs. 1—5).

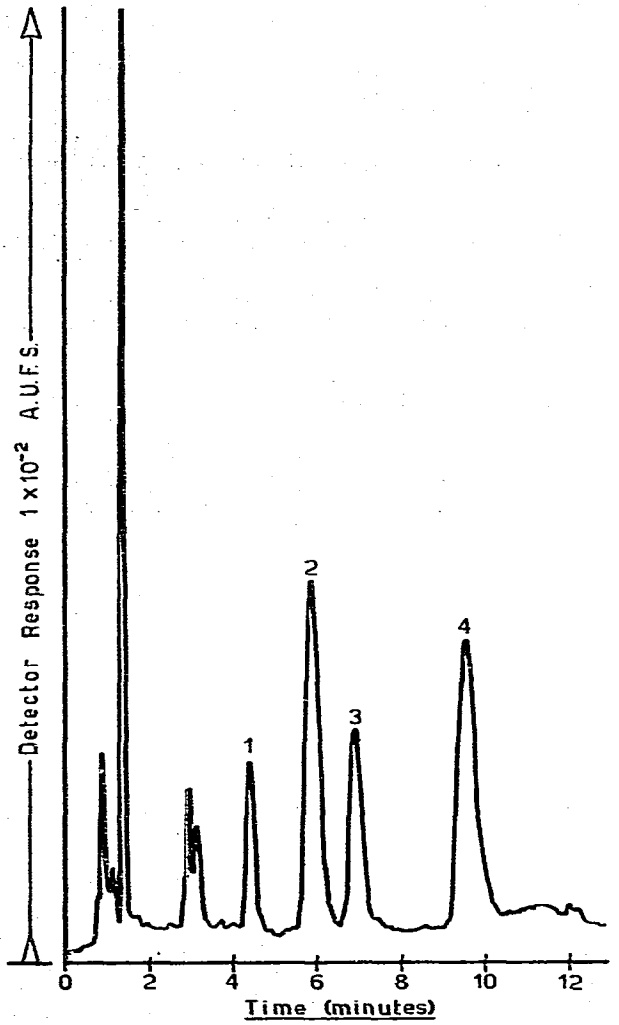

Fig. 5. Chromatogram of a human plasma extract from a patient having received a single oral administration, 30 mg chlordiazepoxide, taken at 36 h. Peaks: 1, demoxepam; 2, desmethylchlordiazepoxide; 3, nitrazepam; 4, chlordiazepoxide. Column, Hibar.

In conclusion, we consider that reversed-phase HPLC is at present the most suitable method of analysis for determining chlordiazepoxide and its metabolites in body fluids because of its specificity, sensitivity, simplicity and speed of execution.

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