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Note

# Simultaneous determination of chlordiazepoxide and its metabolites in human plasma and urine by means of reversed-phase high-performance liquid chromatography

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Chlordiazepoxide (Librium) is widely used for the relief of anxiety, tension and CNS depressions. A number of metabolites of chlordiazepoxide are possible, but so far only four metabolites and the parent compound could be identified in biological fluids.

Methods that have been described for determining the parent compound and metabolites include spectrofluorimetry [1, 2], gas chromatography—mass spectrometry [3, 4], radioimmunoassay [5], thin-layer chromatography [6—8], spectrophotometry [9], colorimetry [10], differential pulse polarography [11] and high-performance liquid chromatography [12—15]. These methods either are laborious or require large sample volumes.

The method described here permits the simultaneous determination of chlordiazepoxide and its metabolites N-desmethylchlordiazepoxide, demoxepam, N-desmethyldiazepam and oxazepam. The small plasma sample volume of 0.2 ml allows frequent sampling from laboratory animals and patients and from volunteers by self-sampling with fingertip puncture. The method has been applied to human pharmacokinetic studies, routine monitoring and cases of severe chlordiazepoxide overdose. Some examples of the pharmacokinetic behaviour of chlordiazepoxide and its metabolites in man will be shown.

## EXPERIMENTAL

### *Apparatus*

A Spectra Physics 3500B high-performance liquid chromatograph equipped with a variable-wavelength detector (Model SP 770), operating at 240 nm was used. The detector was connected to a 10-mV recorder (BD 40, Kipp & Zonen,

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Delft, The Netherlands). A stainless-steel column (10 cm × 4.6 mm I.D.) packed with LiChrosorb RP-8, particle size 5 μm, was used. The injection loop volume was 100 μl.

*Solvent*

The solvent was 0.01 *M* sodium acetate in water—methanol—acetonitrile (600:200:200) and the flow-rate was 2.0 ml/min at a pressure of about 270 bar. All reagents were of analytical-reagent grade and were obtained from Merck (Darmstadt, G.F.R.).

*Drugs*

Chlordiazepoxide, N-desmethylchlordiazepoxide, demoxepam, N-desmethyldiazepam and oxazepam were obtained from Hoffmann-LaRoche (Mijdrecht, The Netherlands).

*Patients*

Samples were taken from patients in the Departments of Neurology and Psychiatry and the Intensive Care Unit of the Sint Radboud Hospital.

*Sample preparation*

*Plasma.* A 10-μl aliquot of water containing 334 ng of diazepam as internal standard and 1 ml of diethyl ether was added to 0.2 ml of plasma and mixed on a Vortex mixer for 1 min. The mixture was then centrifuged for 5 min at 4000 rpm (2600 *g*) in a Heraus Christ centrifuge. The ether layer was removed and evaporated to dryness with a dry stream of air. The residue was dissolved in 0.2 ml of the eluent and 0.1 ml was injected on to the column.

*Urine.* A 0.1-ml urine sample was injected directly on to the column.

*Deglucuronidation of plasma and urine*

*Plasma.* A 0.2-ml volume of plasma was incubated with 15 μl of β-deglucuronidase (100,000 U/ml; Sigma, St. Louis, MO, U.S.A.), 0.2 ml of potassium dihydrogen orthophosphate buffer (0.067 *M*) and one drop of 0.2 *M* acetic acid and 10 μl of water containing 334 ng of diazepam for 16 h. A 100-μl volume of 1 *N* sodium hydroxide solution was added and the mixture was extracted with 1 ml of diethyl ether and treated for injection as described above.

*Urine.* A 0.2-ml volume of urine was incubated with 15 μl of β-deglucuronidase, 0.2 ml potassium dihydrogen orthophosphate buffer (0.067 *M*) and one drop of 0.2 M acetic acid for 16 h. After centrifugation of the mixture, 100 μl were injected directly on to the column.

*Recovery*

The recoveries of the extraction were 95 ± 6% for chlordiazepoxide, 93 ± 3% for N-desmethylchlordiazepoxide, 94 ± 4% for demoxepam, 89 ± 7% for N-desmethyldiazepam and 88 ± 2% for oxazepam (plasma). The detection limit for all derivatives was 20 ng/ml. The calibration graphs were linear for the concentration range 20 ng/ml—20 μg/ml.

## RESULTS

Fig. 1 shows the possible pathways in the metabolism of chlordiazepoxide and the structural formulae of the metabolites that can be expected.

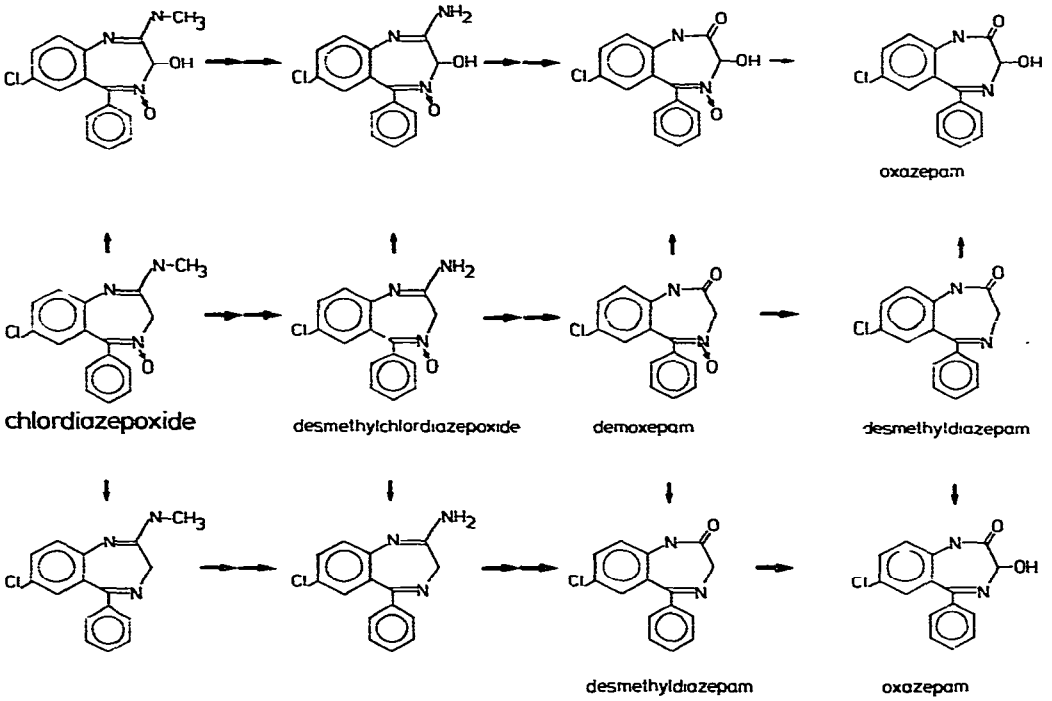


**Fig. 1. Structural formulae of possible metabolites of chlordiazepoxide due to metabolic pathways of N-demethylation + N-deamination, $C_3$ hydroxylation and N → O reduction. Metabolites identified are indicated by their names. The thickness of the arrows indicates the relative rate of each metabolic process.**

Fig. 2 shows the HPLC traces for chlordiazepoxide (peak 4) and its metabolites desmethylchlordiazepoxide (2), demoxepam (1), N-desmethyldiazepam (5) and oxazepam (3) in blank plasma, a standard plasma sample and a plasma sample from a patient receiving 10 mg of chlordiazepoxide (Librium) three times a day. The relative retention times ($k'$) are shown in Table I.

TABLE I

RELATIVE RETENTION TIMES ($k'$) OF CHLORDIAZEPOXIDE AND ITS METABOLITES

| Compound | $k'$ |
|---|---|
| Demoxepam | 6.20 |
| Desmethylchlordiazepoxide | 8.10 |
| Oxazepam | 9.60 |
| Chlordiazepoxide | 11.20 |
| Desmethyldiazepam | 15.20 |
| Diazepam (internal standard) | 22.20 |

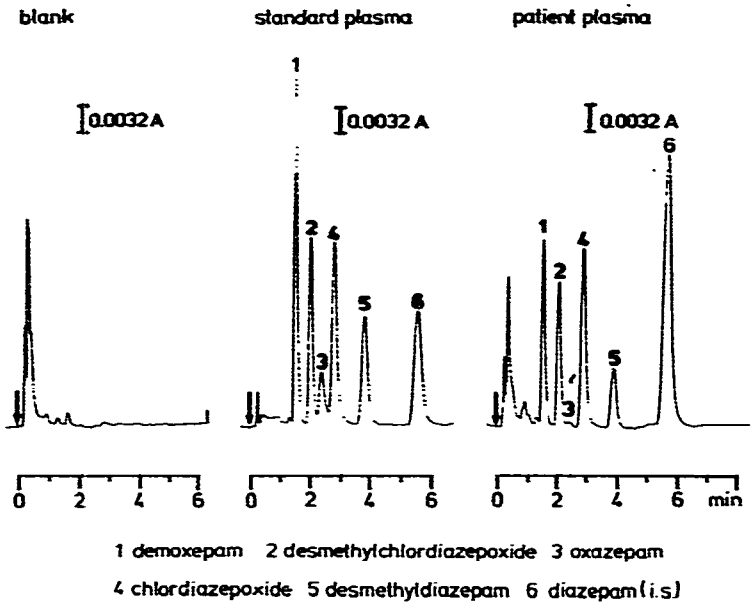


Fig. 2. HPLC traces for chlordiazepoxide (4) and its metabolites N-desmethylchlordiazepoxide (2), oxazepam (3), N-desmethyldiazepam (5) and the internal standard diazepam (6) in a blank human plasma sample (left), in a standard plasma sample (centre) and in a plasma sample from a patient receiving 10 mg of Librium three times a day (right).

Fig. 3 shows the plasma concentration—time profiles of chlordiazepoxide in a patient after ingestion of an apparent overdose of chlordiazepoxide. The plasma concentration of chlordiazepoxide starts with an extremely high value of 12 μg/ml, but declines rapidly with a half-life ($T_{½}$) of 6 h. Chlordiazepoxide is demethylated to N-desmethylchlordiazepoxide ($T_{½}$ 10 h), which in turn is deaminated to demoxepam ($T_{½}$ 28 h); this is subsequently reduced to N-desmethyldiazepam ($T_{½}$ 46 h), which is finally hydroxylated to oxazepam ($T_{½}$ 46 h).

Fig. 4 is an example of the plasma concentration—time profile of chlordiazepoxide and its metabolites in a patient receiving a normal treatment regimen of 10 mg of Librium three times a day. The purpose of the study was to compare the bioavailability of 10 mg of Librium three times a day with 30 mg of Librium CR administered once a day. It was found that the plasma concentrations of the parent drug and the four metabolites, including the low concentrations of the final metabolite oxazepam, can be measured with this HPLC method.

## DISCUSSION

The HPLC method for chlordiazepoxide described here is a step forward towards an HPLC analysis that would make it possible to identify and measure all possible metabolites of chlordiazepoxide shown in Fig. 1 without interferences from endogenous substances as previously described for diazepam [16].

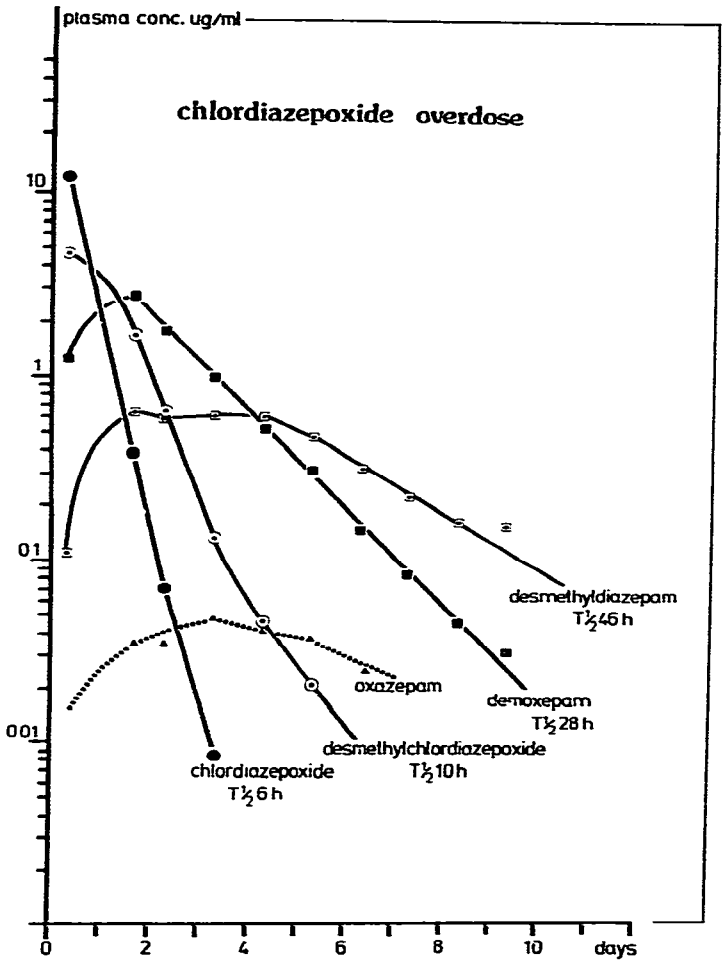


**Fig. 3. Plasma concentration–time profiles of chlordiazepoxide and its metabolites in a patient who had taken an apparent overdose of Librium (about 1 g). The metabolites of chlordiazepoxide, desmethylchlordiazepoxide ($T_{½}$ 10 h), demoxepam ($T_{½}$ 28 h) and desmethyldiazepam ($T_{½}$ 46 h) all have higher $T_{½}$ values than chlordiazepoxide. Oxazepam, with its intrinsic $T_{½}$ of 3 and 10 h, will finally adopt the $T_{½}$ of N-desmethyldiazepam (46 h).**

The advantage of this method over published ones is that, with a simple extraction, chlordiazepoxide and four of its major metabolites can be measured in one run. The small blood sample (0.2 ml of plasma) makes frequent sampling from patients and laboratory animals possible and human volunteers can take blood samples themselves by fingertip puncture. The detection limit of 20 ng/ml facilitates the determination of the plasma concentrations of chlordiazepoxide and its metabolites under conditions of a relatively normal dosage regimen of 10 mg of Librium three times a day, as shown in Fig. 4, and also in samples from patients suffering from an overdose of chlordiazepoxide.

Fig. 3 demonstrates the main metabolic pathway in Fig. 1 from the half-lives of the successive metabolites.

Several HPLC methods have already been developed, but most lack some of

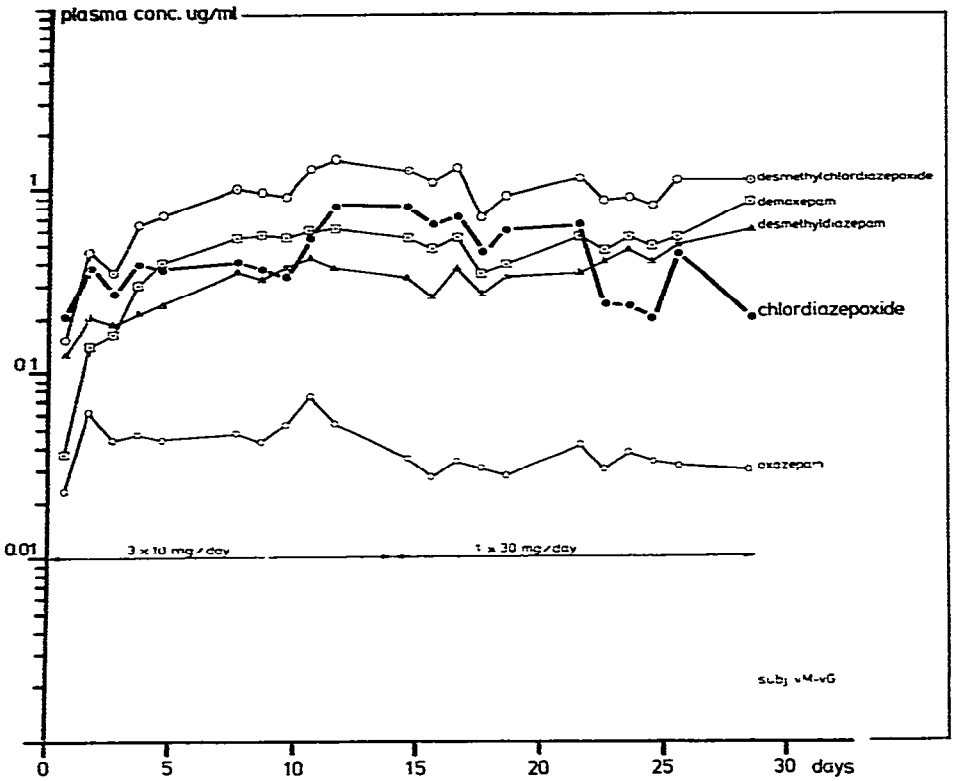


Fig. 4. Plasma concentration—time profile of chlordiazepoxide and its metabolites in a patient treated initially for 14 days with 10 mg of Librium three times a day, replaced in the following 14 days by 30 mg of Librium CR once a day.

the advantages achieved in this work, such as the possibility of showing all metabolites [14, 15]; although while Peat et al. [17], Strojny et al. [13] and Ascalone [12] described suitable HPLC methods, they did not allow the detection of the final metabolite oxazepam.

From the metabolic point of view, the method must contain the possibility of detecting as many metabolites as possible, whether the detection limit allows their actual detection or not. Only when the method of analysis allows the detection of a specific metabolite can one establish whether the metabolite is present in amounts higher than the detection limit or not.

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