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Note

Analysis of chlorodesmethyldiazepam and its metabolites in plasma and urine

L. ZECCA*, P. FERRARIO and R. PIROLA

C.N.R., Centro Studi Fisiologia del Lavoro Muscolare, Via Ampère 56, 20131 Milan (Italy)

and

S.R. BAREGGI

Dipartimento di Farmacologia, Via Vanvitelli 32, 20129 Milan (Italy)

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Chlorodesmethyldiazepam [7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepine-2-one, CDZ, Fig. 1] is a benzodiazepine marketed as En (Ravizza-Milano I), which has been shown to have a powerful anxyolitic effect [1,2]. In previous studies, biotransformation in animals of this compound was demonstrated to proceed almost exclusively via C-3 hydroxylation to lorazepam (LRZ) and further glucuronidation [3,4]. De Silva et al. [3] reported a method for determination of CDZ and LRZ in blood and urine of dogs, administered with CDZ 10 mg/kg i.v. and p.o. Lanzoni et al. [5] described the analysis of CDZ and LRZ in blood and tissues of rats and mice given 5 mg/kg of this drug. The mentioned assays were also applied to human studies, where the CDZ dosage is 0.5–2 mg; this means that much lower blood and urine concentrations of the parent drug and its metabolites are observed [6,7]. These methods suffer from various limitations, such as being time consuming [3] and the use of an inappropriate internal standard [5]; moreover, the determination of lorazepam-glucuronide (LRZ-GLU) in human plasma and urine is not described [3,5,6], and both methods employ a 3% OV-17 packed column, which gives a significant tailing of the CDZ peak.

The purpose of this work was to find an accurate, sensitive and rapid method for analysis of CDZ and its metabolites, to be used in human kinetic and metabolism studies.

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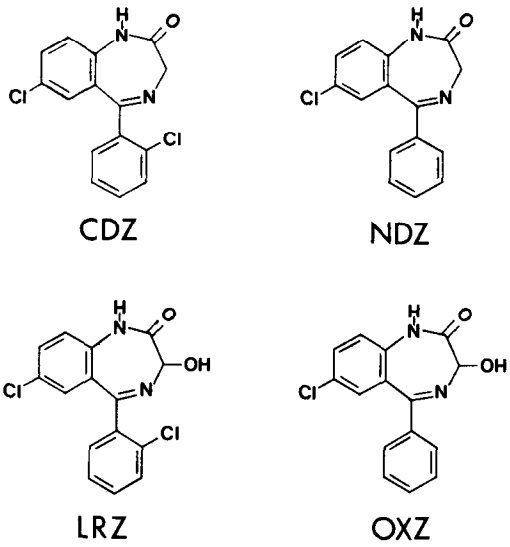

Fig. 1. Structures of chlorodesmethyldiazepam (CDZ), lorazepam (LRZ) and their internal standards nordiazepam (NDZ) and oxazepam (OXZ).

EXPERIMENTAL

Materials

Chlorodesmethyldiazepam, lorazepam, N-desmethyldiazepam and oxazepam were supplied by Ravizza (Muggiò, Italy). β-Glucuronidase–arylsulphatase (glusulase) was obtained from Merck (Darmstadt, F.R.G.). All other reagents and solvents were of analytical grade (Carlo Erba, Milan, Italy).

Stock solutions of benzodiazepines (1 mg/ml) in acetone were prepared weekly and stored at 5°C protected from light. Borate buffer solution was prepared by dissolving 19.5 g of boric acid, 23.5 g of potassium chloride and 19.6 g of sodium carbonate in 500 ml of water. Phosphate buffer (pH 4.5) was 1 *M* potassium dihydrogenphosphate.

Apparatus and conditions

A Perkin-Elmer (Norwalk, CT, U.S.A.) Model Sigma 4 gas chromatograph equipped with a ^{63}Ni electron-capture detector was employed. The system was operated with two different columns: a glass Supelco (Bellefonte, PA, U.S.A.) SPB-5 wide-bore column with SE-54 (30 m×0.75 mm I.D.) with an oven temperature of 240°C and injector and detector temperatures of 280°C; and a 180 cm×4.0 mm I.D. glass column packed with 3% OV-17 on Chromosorb Q (100–200 mesh) also from Supelco, with an oven temperature of 280°C and injector and detector temperatures of 350°C. The flow-rate of carrier gas (argon–methane, 95:5) was 45 ml/min for the packed column and 8 ml/min for the wide-bore column.

Procedure

Plasma CDZ and free LRZ were determined by pipetting 1 ml of plasma into a tube containing 50 ng of nor-diazepam (NDZ) and 20 ng of oxazepam (OXZ) (double internal standard). A 1.5-ml volume of borate buffer, followed by 5 ml of toluene–isoamyl alcohol (100:1.5), was introduced, and the tubes shaken on a reciprocal shaker for 10 min. After centrifuging at 2000 *g* for 10 min, 4 ml of the organic phase were transferred into conical-bottomed tubes and evaporated to dryness at 50°C under a flow of nitrogen. Residues were dissolved in 100 μl of toluene–isoamyl alcohol (100:5), then either 5–7 μl were injected onto the packed column or 1–2 μl were injected onto the wide-bore column.

LRZ-GLU plus unchanged LRZ were measured by the following procedure. To 1 ml of plasma were added the two internal standards, 0.5 ml of phosphate buffer, 40 μl of glusulase (β-glucuronidase 10 U/ml and arylsulphatase 60 U/ml). The solution was then incubated for 20 h at 37°C, before borate buffer was added and the samples were extracted as described above.

Urine CDZ, LRZ and LRZ-GLU were analysed according to the procedure described for plasma. Plasma and urine calibration standards were prepared for CDZ and LRZ, then repeated analyses were done to determine the recovery, precision and linearity of the method. The concentrations of the calibration curve were: for CDZ, 1, 10, 20, 50 and 100 ng/ml and 50 ng/ml of NDZ (I.S.); for LRZ, 5, 10, 20 and 50 ng/ml and 20 ng/ml of OXZ (I.S.). The analysis of LRZ-GLU in plasma and urine was carried out using LRZ as reference standard, since this conjugated compound was not commercially available and a difficult five-step synthesis would be necessary for its preparation.

Blood and urine samples

Venous blood samples were collected in heparinized tubes from patients given 0.5-mg tablets of CDZ twice daily for 5 days. After centrifugation, plasma was separated and stored at −40°C until analysis. Urine samples were collected on the same day (5th) as the blood samples and stored as described for plasma.

RESULTS

Chromatograms of blank plasma and urine extracts are free of peaks that interfere with CDZ, LRZ and the corresponding internal standards (NDZ and OXZ), as shown in Figs. 2 and 3. For comparison, the same plasma extracts were injected onto the wide-bore column. It was observed that the separation obtained with the wide-bore column was better than that resulting from the packed one, and the CDZ and NDZ peaks were more symmetrical. This method was also applied for analysis of CDZ and its metabolites in plasma and urine after single [8] and multiple [9] oral dosing of CDZ. It was observed that CDZ, LRZ and LRZ-GLU are found in plasma after repeated administration (Fig. 2), but no LRZ or LRZ-GLU is found in blood after a single dose [8]. Only LRZ and LRZ-GLU were present in urine after multiple (Fig. 3) and single dosing [8,9].

The concentration of conjugated LRZ was calculated from the difference between the total LRZ obtained by extracting after glusulase incubation and the

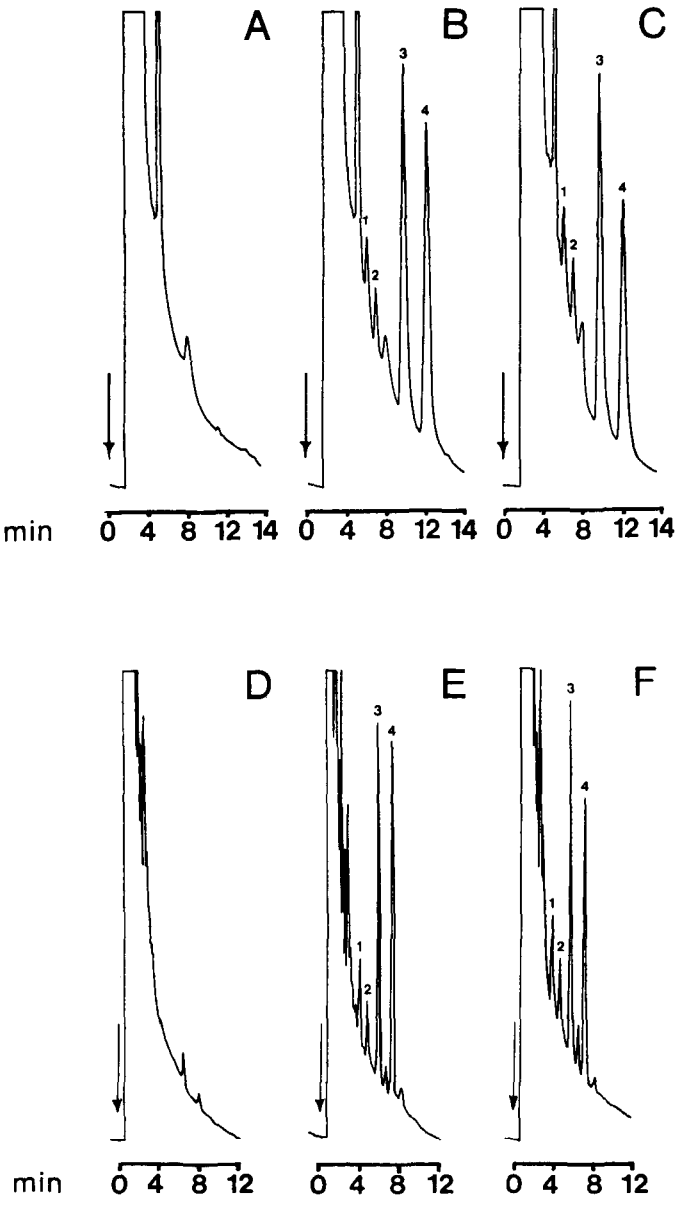

Fig. 2. Chromatograms of plasma extracts obtained with a packed column (see text): (A) human blank plasma; (B) plasma standard containing 20 ng/ml OXZ (1), 5 ng/ml LRZ (2), 50 ng/ml NDZ (3) and 20 ng/ml CDZ (4); (C) plasma sample from a subject given CDZ (0.5 mg) according to conditions given in text; (D), (E) and (F) the equivalent three chromatograms from a wide-bore column.

free one obtained by direct extraction of sample. The recovery of LRZ was demonstrated to be steady after 15–20 h incubation of the samples with glusulase, though it was not possible to calculate the absolute recovery, owing to the lack of an LRZ-GLU standard. This was the only way to assess the completeness of

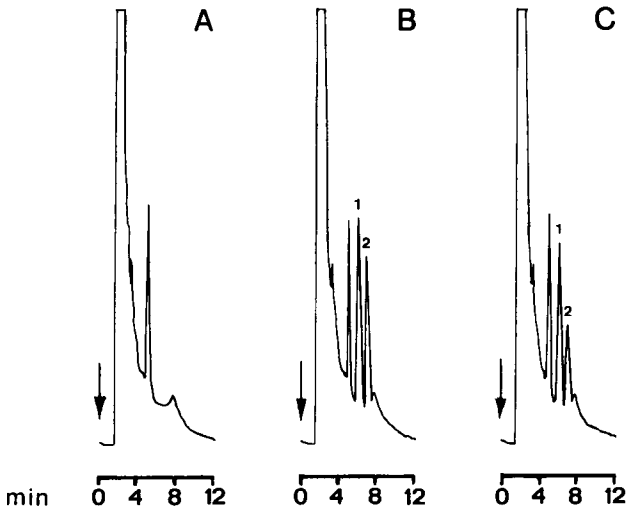

Fig. 3. Chromatograms of urine extracts obtained with a packed column: (A) blank urine; (B) urine standard spiked with 70 ng/ml OXZ (1) and 15 ng/ml LRZ (2); (C) urine sample from a subject given CDZ (0.5 mg) according to conditions given in the text.

enzymatic hydrolysis. Tables I–IV list values for the recovery and coefficients of variation for all components, in intra-day and inter-day assays.

The linearity was assessed for CDZ in the range 0–100 ng/ml (plasma) and for LRZ in the range 0–50 ng/ml (plasma and urine).

The sensitivity of this procedure was 0.2–0.3 ng/ml for CDZ and 1–2 ng/ml for LRZ in both plasma and urine. The detection limit of CDZ was lower than that of LRZ, which decomposed [10] at the column temperatures used.

Even after 30 days with twice-daily administration (1 mg each), there were no measurable peaks of metabolites other than LRZ and LRZ-GLU in plasma or urine [8].

We determined by experiment that plasma and urine samples were stable at

TABLE I

PRECISION, RECOVERY AND DAY-TO-DAY REPRODUCIBILITY OF THE ANALYSIS OF CDZ IN SPIKED HUMAN PLASMA

Intra-day				Inter-day C.V. (%) ($n=5$)
Amount added (ng/ml)	Amount found (mean ± S.D.) (ng/ml)	C.V. (%) ($n=5$)	Mean recovery (%)	
1	1.03 ± 0.04	3.5	103	5.4
10	9.69 ± 0.30	3.1	96.9	4.0
20	19.1 ± 0.63	3.3	95.5	3.4
50	48.6 ± 1.36	2.8	97.2	3.1
100	98.3 ± 2.65	2.7	98.3	2.9

TABLE II

PRECISION, RECOVERY AND DAY-TO-DAY REPRODUCIBILITY OF THE ANALYSIS OF LRZ IN SPIKED HUMAN PLASMA

Intra-day				Inter-day C.V. (%) ($n=5$)
Amount added (ng/ml)	Amount found (mean ± S.D.) (ng/ml)	C.V. (%) ($n=5$)	Mean recovery (%)	
5	4.37 ± 0.24	5.6	87.3	9.3
10	9.04 ± 0.34	3.8	90.4	4.7
20	18.82 ± 0.55	2.9	94.1	4.1
50	48.65 ± 1.61	3.3	97.3	3.5

TABLE III

PRECISION, RECOVERY AND DAY-TO-DAY REPRODUCIBILITY OF THE ANALYSIS OF LRZ IN SPIKED HUMAN URINE

Intra-day				Inter-day C.V. (%) ($n=5$)
Amount added (ng/ml)	Amount found (mean ± S.D.) (ng/ml)	C.V. (%) ($n=5$)	Mean recovery (%)	
5	4.59 ± 0.28	6.2	89.7	9.6
10	9.68 ± 0.39	4.1	97.3	5.3
20	19.46 ± 0.88	4.4	96.4	5.1
50	49.1 ± 1.82	3.7	98.7	4.0

TABLE IV

PRECISION AND DAY-TO-DAY REPRODUCIBILITY OF THE ANALYSIS OF LRZ-GLU IN HUMAN PLASMA AND URINE

Sample	Intra-day		Inter-day	
	Amount found (mean ± S.D.) (ng/ml)	C.V. (%) ($n=3$)	Amount found (mean ± S.D.) (ng/ml)	C.V. (%) ($n=3$)
Plasma	13.7 ± 0.84	6.1	14.0 ± 1.11	7.9
	19.2 ± 1.02	5.3	18.9 ± 1.27	6.7
	16.4 ± 0.85	5.2	16.7 ± 1.14	6.8
Urine	23.8 ± 1.57	6.6	23.2 ± 1.97	8.5
	29.7 ± 1.87	6.3	28.9 ± 2.25	7.8
	35.4 ± 2.19	6.2	34.7 ± 2.53	7.3

−40 °C for at least 7 months, and that the evaporated extracts stored at 5 °C were stable for 2–3 days.

DISCUSSION

The extraction mixture used here allowed us to obtain high recoveries without extracting interfering compounds. Lanzoni et al. [5] used benzene for the extraction, which gives a lower recovery of LRZ. This is quite a disadvantage because this metabolite, which is present only in low concentration, undergoes an on-column thermal rearrangement that results in a low detector response [10,11]. The double extraction procedure applied by De Silva et al. [3] gave very good recoveries, but on the other hand was more time-consuming than our method.

Because the concentration of CDZ in urine is negligible after single or repeated administration of CDZ, we did not include it in our precision and recovery determinations. The use of two internal standards was found very important in achieving good precision. The structures of NDZ and OXZ are closely related to those of CDZ and LRZ, respectively. In particular, OXZ decomposes in the same way as LRZ at the column temperature used, and the chromatographic behaviour of NDZ is very similar to that of CDZ. In the procedure described by De Silva et al. [3], the internal standard used [7-iodo-1,3-dihydro-5-(2′-fluorophenyl)-2*H*-1,4-benzodiazepine-2-one] was reliable for CDZ but not for LRZ analysis; however, this compound is not commercially available. Lanzoni et al. [5] employed pinazepam as internal standard, which is completely unrelated to both LRZ and CDZ, and their results were affected by high coefficients of variation. The extraction buffer used was almost identical with the one introduced by De Silva et al. [3], and later used to extract several benzodiazepines.

The plasma concentrations observed were similar to those reported by Dal Bo et al. [7] for CDZ after a single dose (2 mg); no data were reported for multiple dosing.

A major general disadvantage when analysing N-dealkylated benzodiazepines on packed columns is the persistent tailing, which means worsened separation and non-equilibrium adsorption on the stationary phase. This problem has been solved here by the use of a wide-bore column.

The chromatograms given by De Silva et al. [3] look very similar to ours but very different from the ones reported by Lanzoni et al. [5] and Dal Bo et al. [7]. Surprisingly, identical chromatograms were reported for rat blood [5] and human blood [7] under different chromatographic conditions.

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