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DETERMINATION OF CADRALAZINE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of cadralazine in human plasma and urine. To 1 g of plasma (pH 7) or urine (adjusted to pH 11), internal standard was added and the samples were extracted with chloroform–ethanol (95:5, v/v). The substances were then back-extracted into acid (pH 2) and 100 μl of the aqueous phase were injected. Chromatography was performed on a 10-μm LiChrosorb RP-8 column with acetonitrile–phosphate buffer pH 6 (15:85, v/v) as eluent at a flow-rate of 2.7 ml/min. The substances were detected by UV spectrophotometry at 254 nm. Concentrations down to 0.141 nmol/g in plasma or 10.59 nmol/g in urine could be measured with very good precision. This method was applied to samples from two healthy volunteers given a single oral dose of 10 mg or 20 mg of cadralazine.

INTRODUCTION

Cadralazine, 2-{3-[6-(2-hydroxypropyl)ethylamino]pyridazinyl}ethyl carbazate, is a new, oral antihypertensive vasodilator[1]. To investigate its basic pharmacokinetics in human beings a sensitive assay procedure in human plasma and urine was required.

The high-performance liquid chromatographic (HPLC) method described here makes use of the chromatographic conditions for cadralazine briefly reported by Citerio *et al.*[2] and Crolla *et al.*[3].

EXPERIMENTAL

Reagents

Cadralazine, $C_{12}H_{21}N_5O_3$ (mol. wt. 283.33), was obtained from ISF Laboratories (Trezzano sul Naviglio, Milan, Italy) and the internal standard (CGP 24 751, $C_{13}H_{23}N_5O_3$; mol. wt. 297.35) from Ciba-Geigy (Basle, Switzerland) (Fig. 1). Acetonitrile and dioxane were spectroscopic grade, carbon tetrachloride, chloroform and ethanol of analytical grade (E. Merck, Darmstadt, F.R.G.). Methanol of spectroscopic grade was obtained from Fluka (Buchs, Switzerland). All solvents were filtered through a 0.5-μm FHUP filter (Millipore, Bedford, MA, U.S.A.) before use.

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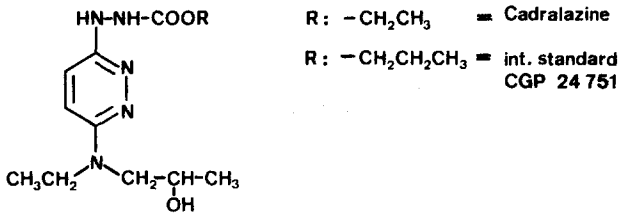

Fig. 1. Structures of cadralazine and the internal standard.

Buffer solutions

Solutions of pH 4 and pH 5 were Titrisol from E. Merck. The other buffer solutions were as follows: pH 2.06 Veibel buffer, 0.090 mol potassium chloride in 1 l of 0.01 mol/l hydrochloric acid; pH 3, 0.050 mol potassium hydrogenphthalate and 0.021 mol hydrochloric acid in 1 l of water; pH 6 phosphate buffer, 0.1 mol $NaH_2PO_4 \cdot H_2O$ (analytical grade, Merck) dissolved in 1 l of doubly distilled water and adjusted to pH 6 with about 30 ml of 1 mol/l sodium hydroxide; pH 12 phosphate buffer, 3.8 g sodium orthophosphate 12-hydrate ($Na_3PO_4 \cdot 12\ H_2O$, analytical grade, Merck) dissolved in 1 l of water and adjusted to pH 12 with about 20 ml of 2 mol/l sodium hydroxide.

10-μm LiChrosorb RP-8 and 30–40 μm Perisorb RP-8 were obtained from Merck.

Fresh calibration solutions of cadralazine were prepared every 3 days in doubly distilled water; the internal standard was dissolved in 0.005 mol/l sulphuric acid and a fresh solution was prepared each week.

Chromatography

The chromatography was performed on a Hewlett-Packard Model 1084 B instrument equipped with a fixed wavelength detector (254 nm) and a variable volume injector. A pre-column was used which consisted of a stainless-steel tube (50 × 3.2 mm I.D.) dry-filled with Perisorb RP-8 (30–40 μm) by the tap-fill method. The analytical column was a stainless-steel tube (250 × 4.6 mm I.D.) filled with 10-μm LiChrosorb RP-8 by the balanced-density slurry packing technique. The slurry, made by dispersing 3.0 g of 10-μm LiChrosorb RP-8 in 30 ml carbon tetrachloride–dioxane (1:1, v/v), was forced into the column by methanol at a pressure of 600 bar.

The mobile phase, acetonitrile–0.1 mol/l phosphate buffer pH 6 (15:85, v/v) was degassed and then used at a flow-rate of 2.7 ml/min. The mobile phase and the column were heated at 30°C. Retention times varied from one column to another, between 5.5 and 6.4 min for cadralazine and 12.0 and 14.3 min for the internal standard.

Sample preparation

Plasma. To 1 g of plasma were added 0.5 ml of internal standard solution (1.682 nmol, 500 ng), 1 ml of doubly distilled water and 6 ml of chloroform–ethanol (95:5, v/v). The mixture was shaken for 12 min at 300 rpm on a horizontal mechanical shaker (Infors, Basle, Switzerland) and centrifuged for 5 min at 1220 *g* on a MSE Multex centrifuge (Crawley, U.K.). The aqueous phase was eliminated by aspiration

and the chloroform–ethanol phase was transferred with a Pasteur pipette into another tube. To the organic phase were added 0.5 ml of buffer pH 2 and the tube was shaken for 12 min at 400 rpm and centrifuged for 5 min at 1220 *g*. The supernatant aqueous phase was pipetted into a plastic vial attached to the automatic sampling system and 100 μl were injected.

Urine. To 1 g of urine were added 0.5 ml of internal standard solution (0.101 μmol, 30 μg), 1 ml of phosphate buffer pH 12 and 6 ml of chloroform–ethanol (95:5, v/v). The procedure was then as described for plasma.

RESULTS AND DISCUSSION

Stability

The stability of cadralazine and the internal standard in organic solvents (dichloromethane, methanol) and in neutral and acidic aqueous solutions was tested. Between the measurements the solutions were stored at 5°C in a refrigerator. In methanol, cadralazine decomposed almost completely within 24 h. In dichloromethane it underwent 20% decomposition in 70 h. Cadralazine was stable in 0.025 mol/l methanolic sulphuric acid (no degradation within 24 days), 0.005 mol/l aqueous sulphuric acid (6% loss after 14 days) and fairly stable in water (20% loss after 13 days).

The internal standard exhibits acceptable stability in methanol (8% loss after 24 days), water and 0.005 mol/l aqueous sulphuric acid (11% and 6% loss after 5 days, respectively).

Extraction

The pH dependence of the extractability of cadralazine and the internal standard (CGP 24 751) from plasma and urine was evaluated. One gram of plasma or urine was spiked with cadralazine, 1 and 10 μg (3.529 and 35.295 nmol/g), respectively, and the internal standard, 0.53 and 9.87 μg (1.782 and 33.193 nmol/g), respectively, and adjusted to different pH values ranging from 2 to 12 using buffer solutions (Table I). The samples were extracted as previously described. The percentage extraction yield was calculated by comparing the peak height obtained by direct injection of a solution of cadralazine or the internal standard with the peak height after extraction. Although for plasma a pH of 10–11 is optimal for extraction, samples were extracted at pH 7 since under basic extraction conditions the plasma components formed emulsions which were difficult to separate. Typical chromatograms of an extract of blank plasma and of plasma spiked with 303 ng (1.069 nmol/g) cadralazine and 526 ng (1.769 nmol/g) internal standard are shown in Fig. 2.

Urine is best extracted at pH 11, yielding samples with a good separation of cadralazine from interfering urine compounds. Fig. 3 shows typical chromatograms of an extract of blank urine and urine spiked with 111 ng (0.392 nmol/g) of cadralazine and 30 μg (0.101 μmol/g) of the internal standard.

If plasma and urine is adjusted to pH 10–12 with diluted sodium hydroxide solution instead of buffer solution, the extraction yield falls to 34.4% for cadralazine and 41.2% for the internal standard from plasma, and to 2.8–1.7% (cadralazine) and to 1.1–0.7% (CGP 24 751) from urine.

TABLE I

EXTRACTABILITY OF CADRALAZINE AND THE INTERNAL STANDARD (CGP 24 751) FROM PLASMA AND URINE AS A FUNCTION OF pH

Resulting pH	*1-g sample adjusted with*	*% extracted*	
		Cadralazine	*CGP 24 751*
Plasma			
2.3	1 ml of HCl/KCl buffer pH 2.06 + 90 µl of 1 mol/l H_2SO_4	2.1	2.9
3.2	1 ml of phthalate buffer pH 3 + 70 µl of 1 mol/l H_2SO_4	4.1	6.6
4.1	1 ml of citrate/HCl buffer pH 4 + 50 µl of 1 mol/l H_2SO_4	6.7	11.3
5.1	1 ml of citrate/NaOH buffer pH 5 + 20 µl of 1 mol/l H_2SO_4	25.6	51.1
7.2	1 ml of water	57.3	66.8
9.0	1 ml of phosphate buffer pH 12 + 50 µl of 2 mol/l NaOH + 12 µl of 1 mol/l H_2SO_4	63.0	73.7
10.5	1 ml of phosphate buffer pH 12	68.4	80.3
Urine			
2.1	1 ml of buffer pH 2.06 + 25 µl of 1 mol/l H_2SO_4	0.2	0.3
3.0	1 ml of phthalate buffer pH 3 + 10 µl of 1 mol/l H_2SO_4	0.9	2.6
4.2	1 ml of citrate/HCl buffer pH 4	4.6	15.4
5.1	1 ml of citrate/NaOH buffer pH 5	25.9	54.4
7.0	1 ml of water + 4 µl of 1 mol/l H_2SO_4	68.1	81.7
10.9	1 ml of phosphate buffer pH 12	59.4	72.8
11.8	1 ml of phosphate buffer pH 12 + 200 µl of 2 mol/l NaOH	52.3	57.4

Calibration graphs

For plasma a calibration graph was established for the concentration range 0.141–1.765 nmol/g (40–500 ng/g) by adding different volumes of an aqueous reference solution to plasma pools. To each sample of 1 g of plasma, 526 ng internal standard (CGP 24 751) in 0.5 ml of 0.005 mol/l sulphuric acid (1.769 nmol/g) were added and then handled as described in the sample preparation. The peak height ratios, H_x, of cadralazine and the internal standard were calculated and plotted (Fig. 4) against the actual concentrations of cadralazine.

In urine, concentrations are much higher and a calibration graph was constructed in the range 10.59–211.77 nmol/g (3–60 µg/g) in the same way as for plasma. Thirty micrograms of internal standard in 0.5 ml of 0.005 mol/l sulphuric acid (0.101 µmol/g) were added to each 1-g sample and the samples worked up as described. Peak area ratios, F_x, were calculated by dividing the area of the cadralazine peak by the area of the internal standard signal and plotted against the actual concentrations of cadralazine (Fig. 5).

The equations of the calibration curves were calculated by least-squares linear regression; correlation coefficient of each curve was 0.9999.

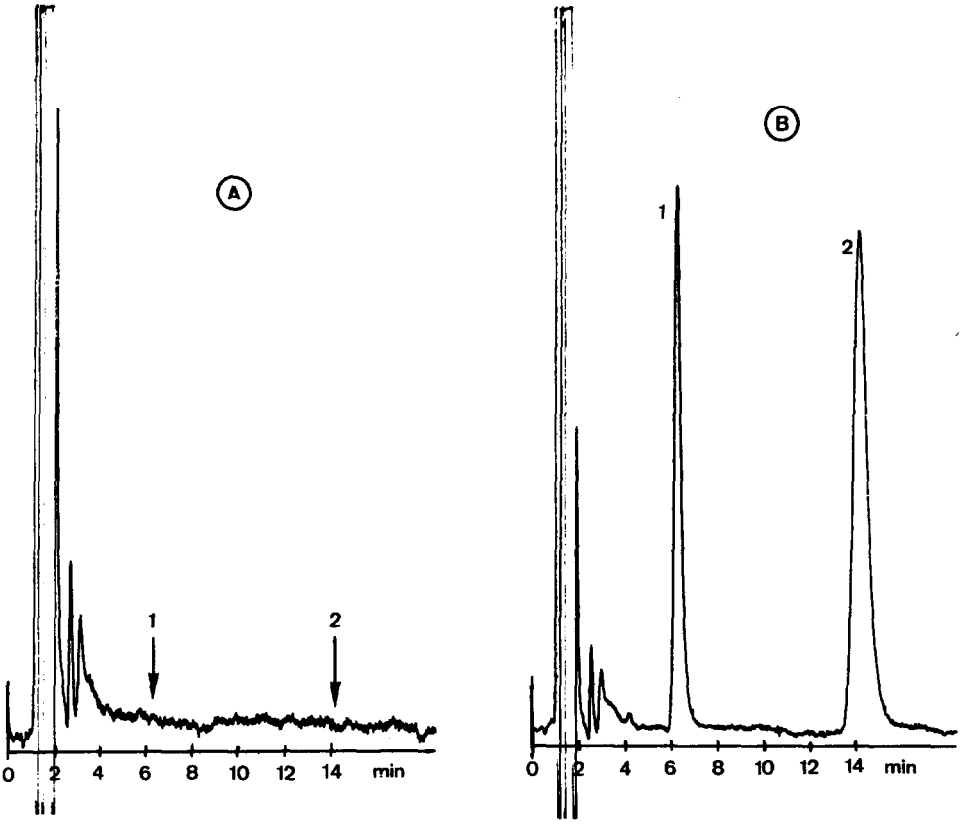

Fig. 2. Chromatograms of an extract of 1 g of blank plasma (A) and 1 g of plasma spiked with 303 ng (1.069 nmol/g) of cadralazine and 526 ng (1.769 nmol/g) of internal standard (B). Peaks: 1 = cadralazine; 2 = internal standard.

Accuracy and precision

The accuracy and precision were evaluated by analysing spiked plasma and urine samples. Eight different concentrations ranging from 0.106 to 1.641 nmol/g (30 to 465 ng/g) for plasma and from 30.00 to 211.77 nmol/g (8.5 to 60 μg/g) for urine, respectively, were analysed in triplicate. The relative standard deviation ranged from 0.2 to 4.0% in plasma and from 0.3 to 5.5% in urine. The deviation of the mean found from the given concentration in plasma ranged from −1.8 to +14.4% and in urine from −1.6 to +1.7% (Table II).

Limit of quantitation

Plasma pools containing 0.141 and 0.035 nmol of cadralazine per g of plasma (40 and 10 ng/g) were prepared and samples of 1 g analysed several times. The relative standard deviation for the determination of 0.141 nmol/g was 1.1% (n = 5) and 22.4% for 0.035 nmol/g (n = 4). Urine samples (1 g) containing 0.392 nmol of cadralazine per g of urine were analysed several times and the relative standard deviation found to be 2.1% (n = 5).

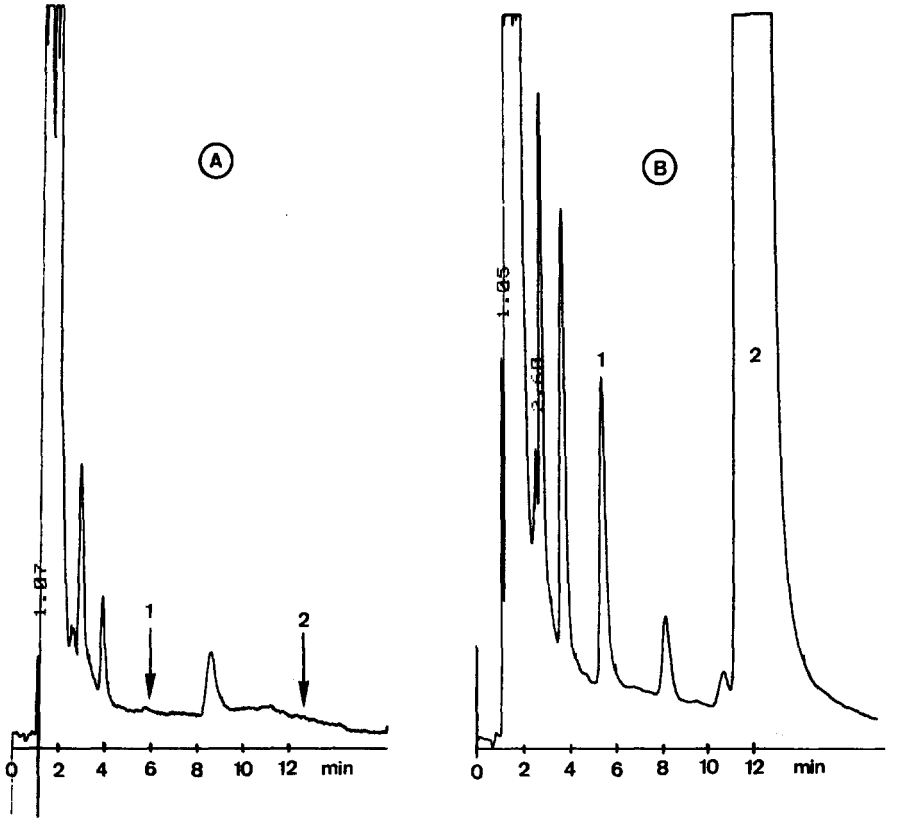

Fig. 3. Chromatograms of an extract of 1 g of blank urine (A) and 1 g of urine spiked with 111 ng (0.392 nmol/g) of cadralazine and 30 μg (0.101 μmol/g) of internal standard (B). Peaks: 1 = cadralazine; 2 = internal standard.

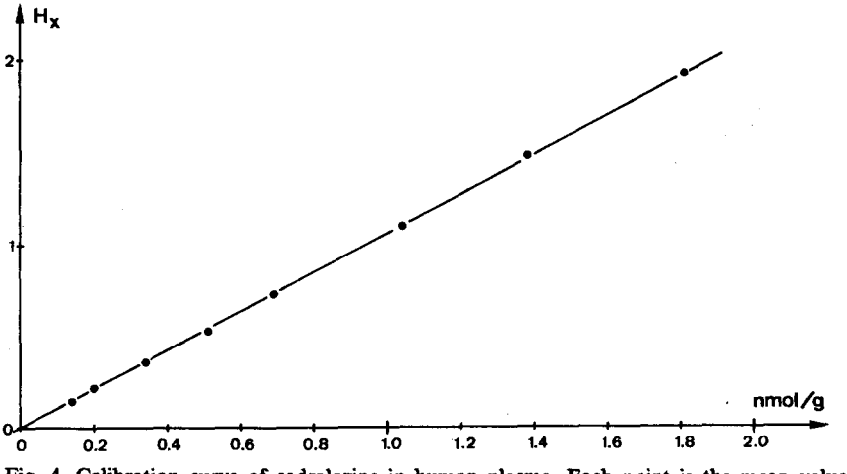

Fig. 4. Calibration curve of cadralazine in human plasma. Each point is the mean value from three measurements.

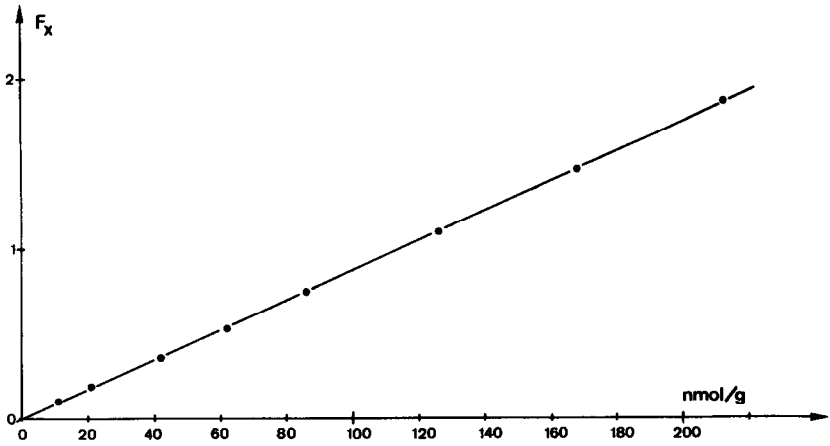

Fig. 5. Calibration curve of cadralazine in human urine. Each point is the mean value from three measurements.

TABLE II

ACCURACY AND PRECISION FOR THE DETERMINATION OF CADRALAZINE IN HUMAN PLASMA AND URINE (n = 3)

Amount introduced (nmol/g)	*Amount found (nmol/g)*	*R.S.D. (%)*	*% deviation from theory*
Plasma			
1.642	1.619	0.84	− 1.4
1.318	1.324	0.63	+ 0.5
0.938	0.927	1.92	− 1.2
0.653	0.641	0.75	− 1.8
0.428	0.433	0.23	+ 1.2
0.265	0.262	1.20	− 1.1
0.159	0.178	2.69	+11.9
0.104	0.119	4.00	+14.4
Urine			
210.23	206.77	0.3	− 1.6
171.88	172.28	1.0	+ 0.2
139.16	139.42	0.4	+ 0.2
121.83	120.68	0.4	− 0.9
79.74	80.57	0.7	+ 1.0
60.78	61.84	5.5	+ 1.7
37.61	37.40	0.3	− 0.6
30.18	29.97	0.5	− 0.7

Application

The applicability of the method for pharmacokinetic studies was tested by analysing plasma and urine samples from two healthy volunteers. The volunteers received a single oral dose of 10 or 20 mg of cadralazine. Plasma and urine samples were collected over 24 h and then analysed for cadralazine. The two plasma profiles

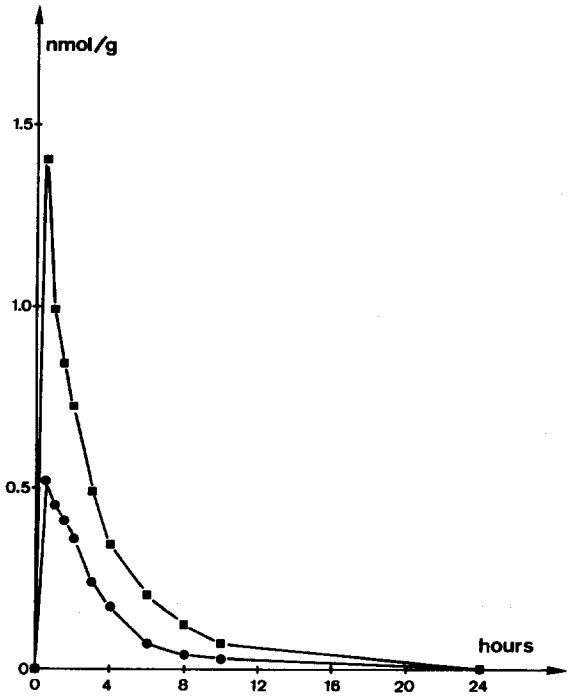

Fig. 6. Plasma levels of cadralazine after a single oral dose of 10 (●) or 20 mg (■) of cadralazine given to two healthy volunteers.

are shown in Fig. 6. The area under the plasma curve after the 10-mg dose was 1.921 (nmol/g) · h and after the 20-mg dose, 4.385 (nmol/g) · h. 79.9% of the 10-mg dose and 71.7% of the 20-mg dose were excreted in the form of unchanged cadralazine in urine within 24 h.

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