Journal of Chromatography, 424 (1988) 119–128
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3956

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

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(First received July 9th, 1987; revised manuscript received September 14th, 1987)

SUMMARY

A sensitive and selective high-performance liquid chromatographic method for determination of intact cadralazine in human plasma or urine has been developed. The sample was buffered (plasma, pH 7.6; urine, pH 12.0) and mixed with internal standard before it was applied to an Extrelut®-3 column. After adsorption, the column was eluted with chloroform, and the eluate was extracted with sodium acetate–hydrochloric acid buffer (pH 2.1). A 20-μl aliquot of the aqueous phase was chromatographed on a 5-μm Spherisorb ODS reversed-phase column, with acetonitrile–phosphate buffer (pH 6.0, 25:75) as eluent. The quantitation was achieved by monitoring the ultraviolet absorbance at 254 nm. The detection limit was 0.03 nmol/ml in plasma and 5.00 nmol/ml in urine. The within-assay variation and the day-to-day reproducibility were $\leq 10\%$ for plasma or urine standard samples. No interferences from possible metabolites or endogenous constituents could be noted. The utility of the method was demonstrated by analysing cadralazine in samples from one hypertensive subject on a therapeutic dose of the drug (7.5 mg orally).

INTRODUCTION

Cadralazine (Fig. 1a) is a new antihypertensive drug, which has shown a long-lasting peripheral vasodilating activity in animals [1] and in humans after oral doses in the range 7.5–30 mg [2–6]. In clinical trials, cadralazine has shown promising results in combination with β-adrenoceptor antagonists and diuretics [3, 4, 6, 7]. The basic human pharmacokinetics of cadralazine were recently reported by Hauffe et al. [8]. In their study, the drug was given in single oral doses to healthy volunteers. The disposition and pharmacokinetics of [^{14}C]cadralazine and its possible metabolites (Fig. 1b–h) in healthy volunteers, following an oral

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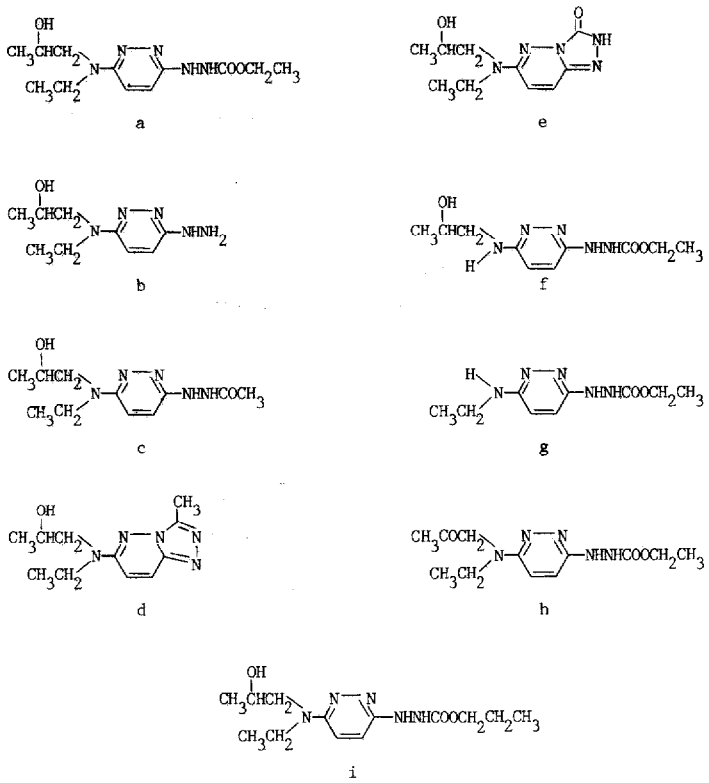

Fig. 1. Chemical structures of cadralazine (a), possible human metabolites (b–h) and internal standard (CGP 24 751; i).

dose, were described by Schütz et al. [9]. In order to make a complete pharmacokinetic characterization of cadralazine, further investigations have to be performed. It is necessary to study the single-dose and the steady-state pharmacokinetics of the drug in hypertensive patients, with and without concurrent medication. Such investigations require a sensitive and selective method for accurate monitoring of plasma and urine levels of cadralazine.

A few high-performance liquid chromatographic (HPLC) methods for determination of cadralazine in biological fluids have been reported [9–13]. The method of Citerio et al. [10] lacks sufficient sensitivity for pharmacokinetic studies. Schütz and Faigle [11] have developed a multiple inverse isotope dilution assay with HPLC separation for the simultaneous determination of [^{14}C]cadralazine and four metabolites (Fig. 1b–e) in rat and dog plasma or urine. This method has been modified recently, to manage the determination of cadralazine and seven possible metabolites (Fig. 1b–h) in human plasma and urine [9]. The method is very sensitive and selective, but it includes time-consuming dilution and extraction steps. The HPLC separation is performed by the use of a gradient system of methanol and phosphate buffer, and the analysis time per sample is very long (ca. 50 min). Another drawback of the method is the requirement for [^{14}C]cadralazine. These disadvantages make the method unsuitable for

routine analysis of the drug. The method of Crolla et al. [13] was not sensitive enough to follow the plasma levels of cadralazine for 24 h after administration of low doses (7.5–10 mg). A time-consuming extraction procedure, an internal standard with a very different chemical structure from cadralazine and the use of an unnecessarily large amount of sample in the assay procedure are examples of other disadvantages of their method. Hauffe and Dubois [12] have made use of the chromatographic observations reported by Citerio et al. [10] and Crolla et al. [13]. The sample preparation is modified, a more appropriate internal standard is used (Fig. 1i), the amount of plasma per sample is reduced, and the sensitivity and precision have been improved. However, it was difficult to reproduce the results when trying their method.

With the aim of finding a selective and sensitive assay for routine analysis of intact cadralazine in human plasma and urine, the HPLC method described in this paper was developed. The method of Hauffe and Dubois [12] has been modified, especially in the extraction step. An increased extraction recovery and a reduced detection limit for cadralazine in plasma were the results. The utility of the method was demonstrated by analysing samples from one hypertensive subject, treated with cadralazine.

EXPERIMENTAL

Equipment

A Milton Roy LDC Constametric III pump, equipped with a Milton Roy LDC Spectromonitor III variable-wavelength UV detector was used. Samples were introduced by syringe into a Rheodyne 7125 injector fitted with a 20-μl loop. A dry-packed precolumn (50 mm × 4.6 mm I.D.; Pellicular media for guard column use with C_{18} groups chemically bonded to the glass beads with a size of 37–53 μm, Whatman, Clifton, NJ, U.S.A.) was used to protect the analytical column – a Spherisorb ODS (C_{18}) reversed-phase column (250 mm × 4.6 mm I.D.; 5 μm particle size; Jones Chromatography, Mid-Glamorgan, U.K.). The chromatograms were recorded and integrated on a Shimadzu C-R3A integrator (Shimadzu, Kyoto, Japan). For sample clean-up a vortex mixer, a horizontal mechanical minishaker (Adolf Kühner, Basle, Switzerland) and glass tubes with screw caps with PTFE facing (ca. 12 ml capacity) were used. Extrelut-3 columns (E. Merck, Darmstadt, F.R.G.) were used for extraction of plasma and urine samples. For centrifugation a Doctor centrifuge 6 (Wifug, Stockholm, Sweden) was used. Glassware was cleaned with 10% nitric acid or chromosulphuric acid and rinsed carefully with distilled water and absolute ethanol before drying and use.

Chemicals and drug standards

All solvents and reagents were of analytical grade. The 0.12 *M* phosphate buffer solution (pH 7.6), the 0.05 *M* sodium acetate–hydrochloric acid buffer (pH 2.1), the 0.10 *M* phosphate buffer (pH 12.0) and the 0.01 *M* phosphate buffer (pH 6.0) were made from commercially available materials (E. Merck) and redistilled water. The buffer solutions were filtered through a Millipore® membrane filter (HAWP; 0.45 μm) before use. Chloroform AR stabilized with 0.6–1.0%

ethanol (E. Merck, No. 2445) and acetonitrile LiChrosolv® for HPLC (E. Merck, No. 1787630) were used. The 0.005 *M* sulphuric acid was made by dilution of concentrated sulphuric acid from E. Merck.

Cadralazine (molecular mass 283.33), the internal standard (I.S.; CGP 24 751; Fig. 1i; molecular mass 297.35) and the metabolites (Fig. 1b–h) were kindly supplied by Ciba-Geigy (Basle, Switzerland).

Standard solutions

A stock solution of cadralazine (175.00 nmol/ml) was prepared by dissolving an accurately weighed sample in redistilled water. A stock solution of the internal standard CGP 24 751 (170.00 nmol/ml) was made by dissolving it in 0.005 *M* sulphuric acid. These solutions were found to be stable for at least one month when stored at 4 °C. Working solutions of appropriate concentrations were made every week by dilution of the stock solutions with redistilled water and 0.005 *M* sulphuric acid, respectively. The calibration solutions contained 0–1.75 nmol/ml cadralazine for the plasma assay, and 0–210.00 nmol/ml cadralazine for the urine assay.

Plasma and urine samples

Drug-free human plasma or urine was used for making the calibration curves. Blood samples from healthy volunteers and hypertensive patients were collected in Vacutainer® tubes for plasma. The blood was centrifuged, and the plasma transferred to plastic tubes fitted with plastic caps. Urine samples were collected in polyethylene bottles. The plasma and urine samples were stored frozen at −20 °C until analysed.

Extraction procedures

Plasma. A 1.00-ml volume of plasma, 0.50 ml of I.S. solution (1.36 nmol/ml) and 2.00 ml of 0.12 *M* phosphate buffer (pH 7.6) were mixed on a vortex mixer in a 10-ml plastic tube. The mixture was then applied to an Extrelut-3 column. After ca. 5 min of adsorption on the support, the column was eluted with 7.00 ml of chloroform. The organic eluate was collected in a 12-ml glass tube and was then mixed with 0.50 ml of 0.05 *M* sodium acetate–hydrochloric acid buffer (pH 2.1). The mixture was gently shaken for 15 min and centrifuged for 5 min at 1370 *g*. The aqueous phase was transferred to a plastic vial. An aliquot of 20 µl was injected into the chromatograph.

Urine. A 1.00-ml volume of urine, 0.50 ml of I.S. solution (170.00 nmol/ml), 1.00 ml of 0.1 *M* phosphate buffer (pH 12.0) and 1.00 ml of redistilled water were mixed in a 10-ml plastic tube. The procedure was then continued as described above for plasma (the organic eluate was mixed with 1.00 ml of 0.05 *M* sodium acetate–hydrochloric acid buffer, pH 2.1, instead of 0.50 ml for plasma).

Chromatographic conditions

A mixture of acetonitrile and 0.01 *M* phosphate buffer (pH 6.0) (25:75, v/v) was used as mobile phase, and it was degassed carefully by sonication before use. The solvent flow-rate was 1.5 ml/min at 154 bar (2300 p.s.i.), and the separation

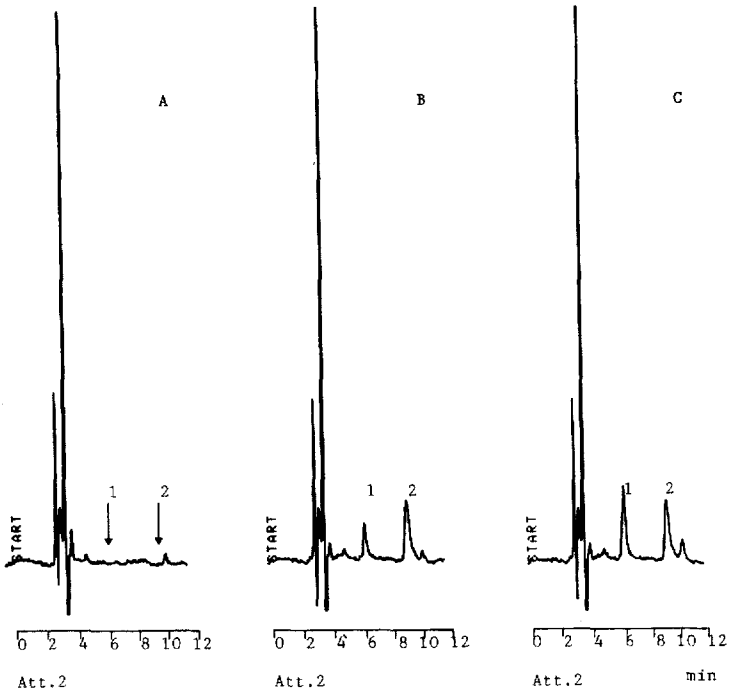

Fig. 2. Chromatograms of (A) plasma blank, (B) plasma blank spiked with cadralazine (0.35 nmol/ml) and I.S. (0.68 nmol/ml) and (C) a plasma sample 1 h after the administration of 15 mg of cadralazine to a patient with hypertension. Peaks: 1 = cadralazine; 2 = I.S.

was performed at room temperature. The total chromatographic time was ca. 15 min per plasma or urine sample. The capacity factors (k') for cadralazine and I.S. were 1.39 and 2.81, respectively. The corresponding retention times were 5.81 and 9.25 min, respectively. The detection wavelength was chosen to 254 nm, where cadralazine and I.S. have their absorption maxima. The chromatograms were recorded with a chart speed of 3.5 mm/min.

Before any sample was injected into the chromatograph, the system was equilibrated with the mobile phase. In order to increase the lifetime of the analytical column, it was protected with a precolumn. The system was also thoroughly cleaned by pumping redistilled water and methanol through the column (15 + 15 min) after each working day.

Typical chromatograms are shown in Fig. 2 for plasma and in Fig. 3 for urine. The peaks are sharp, symmetrical and well defined with respect to the baseline.

Quantitation and calibration curves

The calibration curves were obtained by linear regression of the peak-area ratios of calibration curve standards versus their concentrations of cadralazine, expressed in nmol/ml of plasma or urine. The calibration graphs were constructed on at least five different concentrations in the range 0–1.75 nmol/ml for plasma and 0–210.00 nmol/ml for urine. New calibration curves were established every week. The cadralazine concentration values in samples from volunteers and

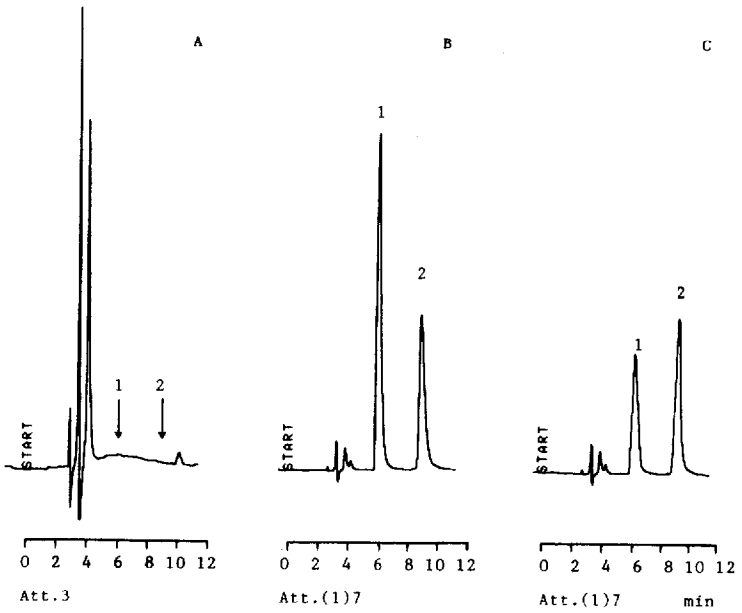

Fig. 3. Chromatograms of (A) urine blank, (B) urine blank spiked with cadralazine (175.00 nmol/ml) and I.S. (85.00 nmol/ml) and (C) a urine sample (fraction 2–4 h after the dose) after the administration of 15 mg of cadralazine to the same patient as in Fig. 2. Peaks: 1 = cadralazine; 2 = I.S.

patients were calculated from the calibration curves. At least two similar samples were assayed in all unknown cases.

RESULTS AND DISCUSSION

Stability of cadralazine and I.S. in solution

The stability of cadralazine and I.S. in different solvents under various conditions of pH, temperature and concentration has been studied [12, 14, 15]. Both cadralazine and I.S. are reported to decompose under certain conditions. However, we did not notice any substantial decomposition of these compounds in our stock solutions. The compounds decomposed to some extent in more dilute solutions, so the working solutions were freshly made every week.

Extraction procedure

Cadralazine is a weak base with a pK_a of 6.0 [13]. The pK_a value of I.S. must be very similar. The extractability of these compounds from plasma and urine into chloroform–ethanol (95:5, v/v) at different pH values was studied by Hauffe and Dubois [12]. Not unexpectedly, the extractability was highest at a pH in the range 7–12 (cadralazine, 52.3–68.4%; I.S., 57.4–81.7%). The introduction of Extrelut extraction columns has made it possible to extract lipophilic compounds in complex media, e.g. drugs in body fluids, with higher recovery, purity and precision, compared with an ordinary liquid–liquid extraction procedure [16]. Another important benefit of the Extrelut extraction method is the saving of time.

TABLE I

HPLC SEPARATION OF CADRALAZINE FROM ITS POSSIBLE METABOLITES AND I.S.

Compound*	Name/code	Retention time (min)	k' value
a	Cadralazine	5.81	1.39
b	CGP22639	3.00	0.24
c	ISF 2876	3.29	0.36
d	ISF 2874	11.30	3.65
e	ISF 3182	3.22	0.32
f	ISF 3472	2.91	0.19
g	ISF 3473	4.20	0.73
h	ISF 3623	10.23	3.20
i	CGP24751	9.25	2.81

*For structures, see Fig. 1.

TABLE II

SUBJECT AND PHARMACOKINETIC DATA FROM THE APPLICATION TEST

C_{max} = maximum cadralazine plasma concentration; t_{max} = time to reach C_{max}; $t_{1/2(\alpha)}$ = elimination half-life for drug in plasma (α-phase); $AUC_{(0-8\,h,s.s.)}$ = area under the curve (Fig. 4a) during 8 h after the dose (steady-state conditions); $Cl_{R(0-8\,h)}$ = plasma clearance of the drug during 8 h after the dose.

Subject data		Pharmacokinetic data	
Age	60 years	Dose	1×7.5 mg orally
Sex	Male	C_{max}	70 ng/ml
Body weight	70 kg	t_{max}	0.5 h
Creatinine (serum)	Normal	$t_{\frac{1}{2}(\alpha)}$	2.2 h
Acetylating capacity	Slow	$AUC_{(0-8\,h,\,s.s.)}$	303 ng/ml h
Acetylisoniazide/isoniazide quote	0.23	$Cl_{R(0-8\,h)}$	265 ml/min
Hypertension duration	6 years	$Cl_{R(0-8\,h)}$/body weight	0.23 l/h kg
Concurrent drug therapy	Metoprolol (Seloken®) 1×200 mg; bendroflumethiazide (Salures®-K) 1×2.5 mg	Cumulative excretion of intact cadralazine (% of the given dose)	
		0-8 h	64%
		0-24 h	81%

With the aim of improving the extraction recoveries of cadralazine and I.S., an appropriate extraction procedure incorporating Extrelut-3 columns was developed.

For the extraction from plasma, the average overall extraction yield of cadralazine was 91%, and of I.S. 80%. For the urine extraction, the corresponding extraction yields were 85 and 78%, respectively. Thus, the extraction yields were substantially improved by the use of Extrelut columns in the first step of the extraction procedure.

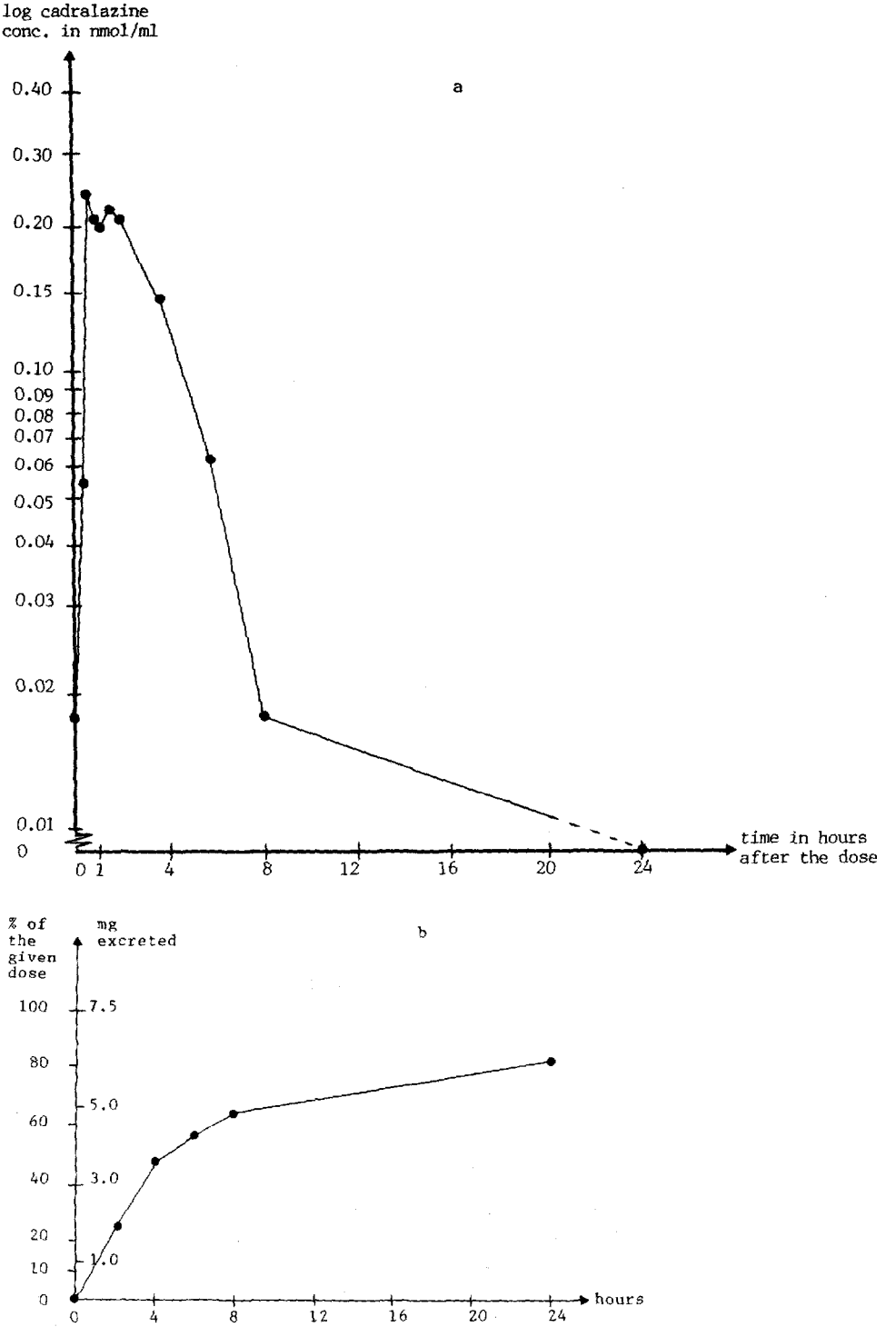

Fig. 4. Plasma concentrations (a) and urinary cumulative excretion (b) of cadralazine, during a 24-h period, obtained for a hypertensive subject after oral administration of 7.5 mg of the drug (steady-state conditions).

Linearity

The equations of the calibration curves were calculated by least-squares linear regression. For plasma: $y = 0.00524x - 0.00057$; correlation coefficient 0.9999; range 0–1.75 nmol/ml. For urine: $y = 0.05141x + 0.00430$; correlation coefficient 0.9999; range 0–210.00 nmol/ml. The ranges of the calibration curves are adequate for the therapeutic levels of cadralazine in plasma and urine, respectively.

Sensitivity

The detection limit (signal-to-noise ratio 2) for cadralazine in plasma was 0.03 nmol/ml and in urine 5.00 nmol/ml, with an injection volume of 20 μl. The relative standard deviations (R.S.D.) at the detection limits were $\leq 10\%$.

Precision

The within-assay precision was studied by calculating the R.S.D. at three different concentrations of cadralazine in plasma and urine, respectively ($n = 10$). For plasma: 8.3% at 0.07 nmol/ml, 5.9% at 0.35 nmol/ml and 3.9% at 1.05 nmol/ml. For urine: 5.1% at 17.00 nmol/ml, 3.5% at 68.00 nmol/ml and 2.9% at 170.00 nmol/ml. The day-to-day reproducibility was obtained by calculating the R.S.D. at 0.07 nmol/ml in plasma and 17.00 nmol/ml in urine ($n = 5$): for plasma 8.1% and for urine 7.0%.

Selectivity

Cadralazine and I.S. were determined with great selectivity by the described method. None of the possible metabolites b–h in Fig. 1 interfered in the chromatograms of plasma and urine samples, as indicated by the retention times and k' values in Table I. Furthermore, no interference was noted from normal endogenous plasma or urine constituents (see Figs. 2 and 3).

Method applicability

The applicability of the method for pharmacokinetic studies was tested by analysing plasma and urine samples from one hypertensive subject (Table II) after repeated oral administration of 7.5 mg (steady-state conditions). Plasma and urine samples were collected over 24 h and then analysed for cadralazine. A representative log (concentration) versus time curve for plasma cadralazine and a plot of the urinary cumulative excretion of intact cadralazine from the patient are shown in Fig. 4a and b, respectively. Pharmacokinetic parameters (Table II) were calculated from the curves: C_{max}, 0.25 nmol/ml; t_{max}, 0.5 h; $t_{\frac{1}{2}(\alpha)}$, 2.2 h; $AUC_{(0\text{-}8\ h,\ s.s.)}$, 1.06 nmol/ml; $Cl_{R(0\text{-}8\ h)}$, 265 ml/min; the cumulative excretion of intact cadralazine (0–24 h), 81% of the given dose. These values indicate that cadralazine is rapidly absorbed and rapidly eliminated as intact cadralazine, mainly by renal excretion.

CONCLUSIONS

The HPLC method described in this report is suitable for monitoring cadralazine in pharmacokinetic studies. The assay is sufficiently sensitive, selective,

rapid and simple to allow accurate and precise measurements of both plasma and urine concentrations of cadralazine under therapeutic conditions.

ACKNOWLEDGEMENTS

I thank Mrs. Katarina Sjöberg, Dr. Kjell Haglund and Dr. Rune Dahlqvist, Departments of Medicine and Clinical Pharmacology, Huddinge Hospital, Karolinska Institute, Stockholm, Sweden, for supplying plasma and urine samples from hypertensive patients, for valuable discussions and kind support. I am indebted to M.Sci. Pharm. Göran Englund, Ciba-Geigy Läkemedel AB, Gothenburg, Sweden, for taking the initiative in the present study, for economic support and fruitful discussions. I am also indebted to Mrs. Inger Lindroth at the Analytical Department, Pharmaceutical Center, Södersjukhuset, Stockholm, Sweden, for skilful technical assistance throughout this work.

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