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DETERMINATION OF CADRALAZINE IN HUMAN WHOLE BLOOD USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: UTILIZING A SALTING-OUT EXTRACTION PROCEDURE

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SUMMARY

A rapid, selective, and sensitive ion-paired reversed-phase high-performance liquid chromatographic method for determination of the new carbazate type of antihypertensive vasodilator agent cadralazine in human whole blood has been developed. Cadralazine was extracted from whole blood by adding 0.5 ml of acetonitrile to 1.0 ml of whole blood followed by salting-out of acetonitrile by the addition of potassium carbonate in excess. An aliquot of the salted-out acetonitrile was injected into the chromatographic system. A column packed with 3-μm octyl (C_8) particles was used with an isocratic elution of 1% acetic acid and 5 m*M* hexanesulfonic acid–acetonitrile (70:30, v/v). The cadralazine was measured using ultraviolet detection at 250 nm and the assay was completed in less than 20 min and had a limit of quantitation of 10 ng/ml for a 100-μl injection volume.

INTRODUCTION

Cadralazine, 2-{3-[6-(2-hydroxypropyl)ethylamino]pyridazinyl} ethylcarbazate, is a new carbazate type antihypertensive vasodilator and has been shown to be quite effective in reducing blood pressure [1,2]. Cadralazine has a long-lasting activity in humans and animals at relatively low dosages [3–6]. Cadralazine has also shown promising therapeutic results when administered concurrently with β-adrenoceptor antagonists and certain diuretics [7–9]. The basic pharmacokinetics of cadralazine in humans has been studied by Schutz et al. [10] and Hauffe et al. [11]. In order to conduct more extensive and complete pharmacokinetic

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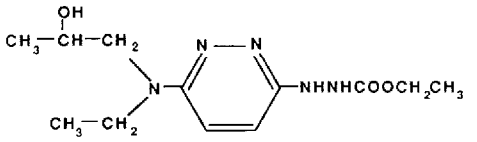

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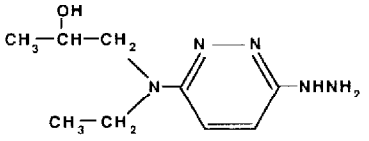

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Fig. 1. Structures of cadralazine (a) and its hydrazino metabolite (b).

study of cadralazine in humans, a reproducible, sensitive, rapid and specific analytical method for measuring the drug accurately in human whole blood, plasma or urine is required.

Determination of cadralazine in human biological samples using high-performance liquid chromatography (HPLC) with ultraviolet (UV) and ^{14}C-radioactivity detection has been reported [12–16]. The sample preparation in most of these methods involves multiple-step extraction, or evaporation of the extracting solvent and dissolution of the residue in mobile phase solution or both This makes the prechromatography isolation of cadralazine from biological samples time-consuming and complicated. Most of the methods also lack the sensitivity for pharmacokinetic studies after a single dose.

In this paper, cadralazine was isolated by a single-step acetonitrile extraction from whole blood samples followed by salting-out of the acetonitrile by anhydrous potassium carbonate salt. The use of ion-pair chromatography increased the selectivity of the parent drug significantly. Using this method, one can quantitate cadralazine in human whole blood down to at least 10 ng/ml for a 100-μl injection volume of the salted-out acetonitrile solution used for extraction. Chemical structures of cadralazine and one of its major metabolites, hydrazino cadralazine, are shown in Fig. 1.

EXPERIMENTAL

Equipment

A Perkin-Elmer Series B-410 solvent delivery system (Perkin-Elmer, Norwalk, CT, U.S.A.) was used to pump the mobile phase through he chromatographic system. The chromatographic system was equipped with a Rheodyne 7275 sample injector with a 200-μl sample loop (Rheodyne, Cotati, CA, U.S.A.). The UV–visible detector used was a Kratos Spectroflow 783 equipped with a flow cell

of 8.0 mm path length (ABI Analytical, Kratos Division, Ramsey, NJ, U.S.A.). The chromatograms were recorded on a Houston Instrument Omniscribe Series D5000 strip chart recorder (Houston Instruments, Austin, TX, U.S.A.). The slurry packer was purchased from Micromeritics (Norcross, GA, U.S.A.) and was used to pack the analytical column. The mobile phase was degassed by a Branson Model 2200 sonicator (Branson Cleaning Equipment, Shelton, CT, U.S.A.). All water used was deionized and was obtained from a Milli-Q system (Millipore, Milford, MA, U.S.A.).

Samples were centrifuged by an IEC centrifuge, Model HN (Damon, IEC Division, Needham Heights, MA, U.S.A.). A C-130B Upchurch guard column dry packed with 10-μm octyl particles was used throughout (Upchurch Scientific, Oak Harbor, MA, U.S.A.)

Materials

The aged, pooled whole blood and plasma was obtained from the Blood Center of Southeastern Wisconsin (Milwaukee, WI, U.S.A.). The reversed-phase 3-μm octyl stationary phase was purchased from Alltech Assoc. (Deerfield, IL, U.S.A.). Cadralazine (CGP-18684) and hydrazino cadralazine (CGP-22639), a metabolite of cadralazine, were kindly provided by Ciba-Geigy (Basel, Switzerland). Acetic acid and hexanesulfonic acid were purchased from Aldrich (Milwaukee, WI, U.S.A.). All solvents and reagents were used as received without further clean-up or purification.

Preparation of stock solution

The stock solution of cadralazine was prepared by dissolving 40 mg of cadralazine in 10 ml of acetonitrile and 1% acetic acid. A 1-ml volume of this solution was diluted to 50 ml in pure acetonitrile solution. Both the solutions were used to prepare whole blood and acetonitrile standards in order to construct the calibration curves.

Chromatographic conditions

The mobile phase consisted of solvent A (100% acetonitrile)–solvent B (5 m*M* hexanesulfonic acid in 1% aqueous acetic acid) (30:70, v/v). The mobile phase was delivered through the chromatographic system at a flow-rate of 1.0 ml/min. Cadralazine and its metabolite hydrazino cadralazine were monitored using UV detection at a wavelength of 250 nm and 1.0–0.005 a.u.f.s. The chromatographic system was operated at room temperature ($25 \pm 2°C$). Quantitation of cadralazine in whole blood samples of a subject was conducted by comparing the peak height of the cadralazine in the sample with a standard calibration curve of cadralazine in human whole blood.

Extraction procedure

The aged whole blood was kept frozen at $-25°C$ and thawed at room temperature before analysis. A 1-ml volume of thawed whole blood was pipetted into a disposable borosilicate culture tube and fortified with an aliquot of a standard stock solution of cadralazine. This was mixed on a vortex mixer for 30 s and 0.50

ml of acetonitrile was added to the mixture. After mixing, the culture tube was centrifuged for 2 min at 2200 *g*. The supernatant was decanted into a fresh test tube and was saturated with anhydrous potassium carbonate (approximately 2000 mg per ml of whole blood). This solution was centrifuged for 3 min at 2200 *g*. The acetonitrile was salted-out at this stage. This acetonitrile was transferred into another fresh test tube. A 100-μl volume of this solution was injected into the HPLC system.

Slurry packing of the analytical column

The analytical column (150 mm × 4.6 mm) was slurry packed using a procedure described elsewhere [17]. The solvent used for slurry packing was methanol–acetic acid–isopropanol (30:20:50, v/v).

Construction of calibration curve

The stock solution of cadralazine described in *Preparation of stock solution* was stored at −25 °C and was found to be stable for at least thirty days. At least five solutions of cadralazine were prepared by adding adequate stock solution in aliquots of whole blood to give concentrations of 0.01, 0.05, 0.20, 0.80 and 1.5 μg/ml.

If the whole blood concentration of cadralazine in patients samples exceeds 1.5 μg/ml, then additional higher-concentration solutions can be used to construct the calibration curve. The calibration curve was linear to at least 10 μg/ml. A straight calibration curve of peak heights versus concentration of cadralazine was constructed and was used for quantification of cadralazine in human whole blood.

Salting-out of acetonitrile from aqueous solution

The procedure for salting-out acetonitrile from aqueous solution using potassium carbonate has been described elsewhere [17].

Stability study of cadralazine in plasma

Pooled plasma (50 ml) was thawed at room temperature and was spiked with standard cadralazine solution at 0.50 μg/ml. This solutions was transferred in approximately 1-ml volumes into borosilicate culture tubes. A 1-ml volume of this solution was analyzed immediately, and this was considered to be the zero-time concentration. It was assumed that no degradation occurred during spiking, mixing or the extraction processes. At different time intervals, another test tube contents was analyzed. These samples were stored at room temperature and the determination was continued for 25 days.

An identical procedure was used for the samples stored at −10 °C. However, at least 2 h were required for the frozen samples to thaw at room temperature. The determination of cadralazine was done immediately after thawing the samples. The kinetic study at −10 °C may not be very accurate because the time required to thaw the samples was not included in the sampling time.

RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram of whole blood containing no cadralazine. Fig. 3 is a chromatogram of whole blood containing cadralazine. The samples were treated by acetonitrile in the extraction procedure. From Fig. 1 it is clear that the retention time region for cadralazine is free of interfering peaks from whole blood. The total time, starting from pipetting the whole blood until the elution of cadralazine, was less than 20 min.

Peak heights were used instead of peak areas for cadralazine determination. Calibration curves were straight line ($r = 0.999$) with negligible y-axis intercepts. From statistical calculations at the 95% confidence interval, the slopes of the calibration curves that are obtained after extraction of cadralazine from whole blood and cadralazine in acetonitrile with no extraction were identical. This result indicates that the steps in the extraction procedure did not enhance or reduce the cadralazine peak heights. Therefore, a calibration curve constructed from a standard solution of cadralazine in acetonitrile may be used to quantitate cadralazine in blood samples of humans who have ingested the drug.

The precision and accuracy of the method were determined by multiple analyses of whole blood samples spiked with a known amount of cadralazine with a concentration between 0.05 and 2.0 μg/ml. Table I shows the assay reproducibility for same-day analyses and Table II shows the reproducibility for day-to-day analyses. The relative standard deviation (R.S.D.) varied from 2.1 to 5.5% for same-day analyses. The results of these analyses indicate that the R.S.D. values

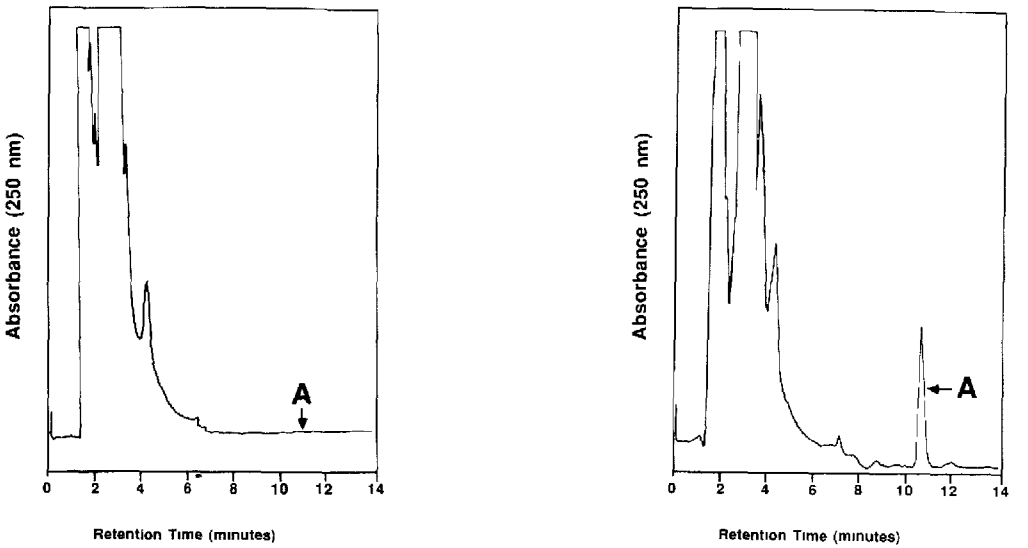

Fig. 2. Chromatogram of human whole blood with no cadralazine. A indicates the retention time of cadralazine. Detector, 250 nm and 0.005 a.u.f.s , injection volume, 100 μl.

Fig 3. Chromatogram of human whole blood for cadralazine. A indicates the cadralazine peak. Detector conditions were the same as in Fig 2 Injection volume, 100 μl; concentration of cadralazine detected, 123 ng/ml.

TABLE I

ASSAY REPRODUCIBILITY FOR THE SAME DAY

Actual concentration (μg/ml)	Concentration determined (mean $\pm$ S.D., $n=8$) (μg/ml)	Relative standard deviation (%)
0.05	0.06 $\pm$ 0.0033	5.5
0.50	0.42 $\pm$ 0.018	4.3
1.00	1.1 $\pm$ 0.023	2.1
2.00	1.8 $\pm$ 0.04	2.3

TABLE II

ASSAY REPRODUCIBILITY FROM DAY TO DAY

Day-to-day analysis was done on every other day for five days.

Actual concentration (μg/ml)	Concentration determined (mean $\pm$ S.D., $n=8$) (μg/ml)	Relative standard deviation (%)
0.05	0.043 $\pm$ 0.0028	6.5
0 50	0.63 $\pm$ 0.024	3.8
1.00	0.85 $\pm$ 0.023	2 7
2.00	2.30 $\pm$ 0.034	1.4

TABLE III

EXTRACTION EFFICIENCY OF CADRALAZINE FROM WHOLE BLOOD

Concentration (μg/ml)	Percentage extracted (mean $\pm$ S.D., $n=5$)			
	Methanol	Ethanol	Isopropanol	Acetonitrile
0.05	97 $\pm$ 3	88 $\pm$ 4	82 $\pm$ 5	96 $\pm$ 5
1.50	96 $\pm$ 4	83 $\pm$ 3	83 $\pm$ 3	95 $\pm$ 5
5.0	97 $\pm$ 4	88 $\pm$ 3	84 $\pm$ 5	97 $\pm$ 3

for the same-day and day-to-day analyses are better at higher concentrations of cadralazine.

The extraction efficiency of cadralazine from whole blood was conducted using different organic solvents using the procedure described in the previous section. The data obtained from this experiment are shown in Table III. With respect to extraction efficiency and selectivity, acetonitrile showed the best results.

The recovery of cadralazine from human whole blood was determined by adding an aliquot of cadralazine standard solution to the whole blood samples, resulting in twice the concentration of cadralazine that had been quantitated before. After spiking, the whole blood samples were treated identically as before.

The concentration was determined by injecting an aliquot of the salted-out extracting solvent onto the HPLC system. The recovery of cadralazine ranged from 91 to 105% over a concentration range of 0.12–0.72 μg/ml.

The selectivity of cadralazine in terms of chromatography was enhanced using an ion-paired reversed-phase technique instead of regular reversed-phase HPLC. The presence of the ion-pairing agent (hexanesulfonic acid) increased the retention time of the drug significantly under the chromatographic conditions used in this experiment. Hexanesulfonic acid did not cause any significant enhancement of the retention times of the whole blood endogenous peaks. The capacity factor (k') of cadralazine decreased with increasing ionic strength of the mobile phase. This effect leveled off when the concentration of sodium chloride in the mobile phase was about 30 m*M*.

The injection volume used in this experiment was 100 μl. No significant distortion of cadralazine peak was observed due to the injection of 100 μl of strong solvent into the HPLC system. It has been shown that the acetonitrile from aqueous media can be salted-out at a volume of 98 ± 4% when different ratios of acetonitrile and water are used [17].

The stability of cadralazine in human plasma at 25 ± 2°C and −10°C was conducted using the chromatographic procedure described in this experiment. Cadralazine degraded at both 25 and −10°C. The semi-natural log plots of (ln of percent parent versus time) gave straight lines (Fig. 4). The data were plotted assuming that the rate of degradation followed first-order kinetics. The degradation rate constant at 25 ± 2°C and −10°C was calculated to be −0.031 and −0.0076 per day, respectively. The calculated degradation half-lives for the above temperatures were 22 and 91 days, respectively. These data reveal that the drug degrades relatively fast at room temperature. Therefore, for accurate results, samples should be analyzed on the same day that they are withdrawn and then frozen at −10°C immediately.

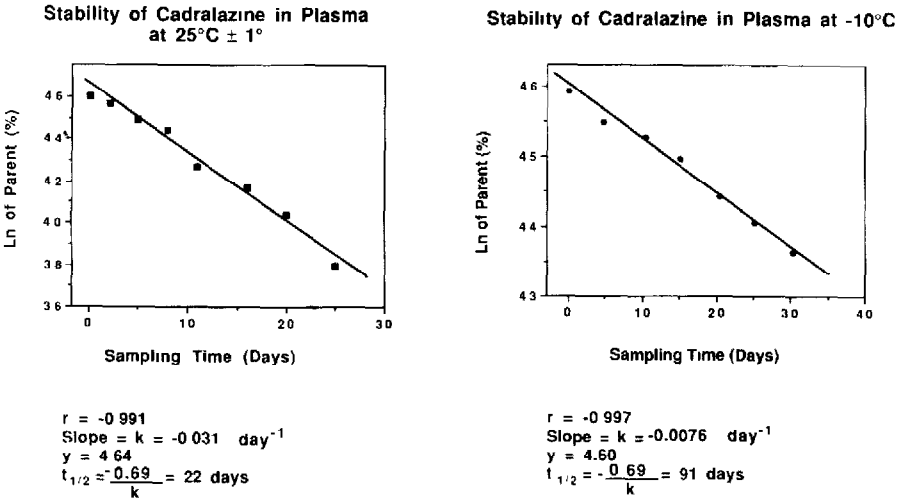

Fig 4. Semilog plot of percent cadralazine remaining versus time for stability study of the parent drug in human plasma at 25 ± 2°C and −10°C.

The HPLC method described in this experiment can be used to conduct the in vivo pharmacokinetic studies of cadralazine in humans. The limit of quantification of the method is about 10 ng/ml for a 100-μl injection volume of the extracting solvent. Interference of some common prescription and non-prescription drugs was also tested; erythromycin, cimetidine, ranitidine, aspirin, acetaminophen, creatinine, theophyline and caffeine all had negative results.

CONCLUSION

The reversed-phase HPLC method described here is simpler and faster than any previous method for cadralazine described in the literature. Using the salting-out extraction procedure with no evaporation or derivatization step increased the sensitivity, reproducibility, and accuracy of the method significantly. Because of the high sensitivity of the method, pharmacokinetic studies of cadralazine can be conducted in humans at very low levels of the drug using this method.

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