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Note

Liquid chromatographic analysis of a new antihypertensive agent, 3-{4-[4-(3-methylphenyl)-1-piperazinyl] butyl}-2,4-imidazolidinedione, in plasma

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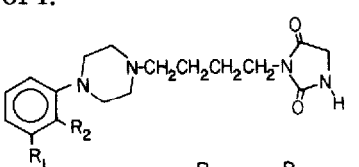
3-{4-[4-(3-Methylphenyl)-1-piperazinyl] butyl}-2,4-imidazolidinedione (I, CI-926, Fig. 1), is a new orally active antihypertensive agent currently undergoing clinical evaluation. Compound I has proven α_1 adrenoceptor blocking activity which is in part responsible for its antihypertensive mechanism of action [1, 2]. A selective assay capable of quantifying the drug at low nanogram per milliliter concentrations was needed for the development of I.



	R_1	R_2
I	$-CH_3$	-H
IS	-H	$-CH_2CH_3$

Fig. 1. Structure of compound I and internal standard (IS).

This report describes the development and validation of an assay method employing liquid—solid extraction of I from human plasma onto Sep-Pak C_{18} cartridges, liquid chromatographic separation on unmodified silica gel using an aqueous—organic mobile phase, and detection by ultraviolet absorption. The basis of the method was established using rat and dog plasma during pre-clinical development [3, 4] and was extended as described here to human plasma.

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EXPERIMENTAL

Reagents

Compound I and the internal standard (I.S.), 3-{4-[4-(2-ethylphenyl)-1-piperazinyl] butyl}-2,4-imidazolidinedione hydrochloride, were synthesized at Warner-Lambert (Ann Arbor, MI, U.S.A.) and were used without further purification. Hexane, dichloromethane, acetonitrile, methanol, and water were HPLC grade. Tetrabutylammonium (TBA) hydrogen sulfate, sodium acetate, glacial acetic acid, and dibasic ammonium phosphate were reagent quality or better. Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.) and used in a Sep-Pak cartridge rack.

Preparation of standards

Calibration standards containing 2, 5, 10, 50, 100, 200, and 500 ng/ml were prepared daily by adding 100-μl aliquots of a serially diluted stock solution (1.0 mg/ml) of I in 0.01 *M* sodium acetate buffer (pH 4.2) to 2 ml of blank plasma. Control standards containing 5, 50, and 200 ng/ml were prepared in 50 ml of blank plasma and were frozen in 2.5-ml aliquots until used. The control standards were analyzed daily with the calibration standards throughout validation and sample analysis periods.

The I.S. solution contained 200 ng per 50-μl aliquot and was prepared by dilution of an aqueous stock solution (0.4 mg/ml). Stock solutions for drug and I.S. were prepared daily.

Extraction

A 2-ml sample, calibration standard or control standard, 50 μl of I.S. solution, and 2 ml of water were combined in each tube and vortexed gently to mix. Sep-Pak cartridges were pretreated with 5 ml of methanol followed by 5 ml of 0.01 *M* tetrabutylammonium hydrogen sulfate, both pulled through the cartridge under 75 mmHg vacuum. After turning the vacuum off, samples were transferred to the Sep-Pak cartridges and pulled through under 75 mmHg vacuum. Immediately after the sample had entered the cartridge it was washed with 5 ml of water without breaking the vacuum. Next, the vacuum was increased (500 mmHg) and the cartridge was allowed to dry for 6 min. The vacuum was turned off and 2 ml of hexane were added to each cartridge and pulled through under 75 mmHg vacuum. The Sep-Pak cartridge was allowed to dry for 3 min under 500 mmHg vacuum after the hexane wash. All of the above solutions were discarded. Dichloromethane (3 ml), was pulled through each cartridge under 25 mmHg vacuum and collected in a clean tube. Each tube of eluate was frozen for about 10 s in a dry ice—methanol bath. The organic layer was transferred to a 12-ml tapered centrifuge tube and evaporated to dryness in a 50°C water bath under nitrogen flow. The residue was dissolved in 100 μl of 0.01 *M* acetate buffer (pH 4.2) by vortex-mixing. Each tube was centrifuged briefly to localize the sample for transfer to an injection vial. An 80-μl aliquot of the sample was injected onto the column.

Plasma recovery

Extraction recovery was determined by spiking blank plasma to contain

25 or 100 ng/ml I or 200 ng/ml I.S. (the working concentration) and extracting as described. Detector response for the extracted samples (n = 6) was compared with that from the injection of the same volume of the stock solution used to prepare the samples.

Column liquid chromatography

Apparatus. The high-pressure liquid chromatographic (HPLC) system consisted of a Hewlett-Packard Model 1090 liquid chromatograph (Avondale, PA, U.S.A.), a Kratos Spectroflow 773 absorbance detector set at 247 nm (Ramsey, NJ, U.S.A.), and a Hewlett-Packard Model 3390A integrator. A Hewlett-Packard Model 1040A scanning diode-array detector was used to determine peak homogeneity while validating the method. The Kratos detector was used for routine sample assay.

Column. Separations were performed on a Brownlee MPLC 5-μm spherical silica column (350 × 2.1 mm I.D.). The column consisted of one 30-mm, one 100-mm, and one 220-mm MPLC cartridge, assembled in sequence using an MPLC cartridge system holder (Santa Clara, CA, U.S.A.). A matching 30 × 2.1 mm I.D. cartridge placed between the pump and injector served as a presaturator. The system was operated at room temperature.

Mobile phase. The mobile phase was acetonitrile–4 m*M* dibasic ammonium phosphate (50:50) mixed by the solvent delivery system. The apparent pH of the mobile phase was 8.3. Both components were degassed by continuous helium sparging. The flow-rate was 0.35 ml/min, which yielded a pressure of approximately 165 bar at the column head.

Statistical analysis

The method was calibrated for each sample set by regressing I/I.S. peak-height ratios against the concentrations of I in the calibration standards. The straight line of best fit was estimated using linear regression with a weighting factor of 1/concentration^2. Concentrations of I in unknown samples were calculated using the regression equation.

Validation of the method was performed by assaying triplicate sets of calibration and control standards on three separate days. The calibration standards from each day were fit with a straight line as detailed above. The daily calibration curves were used to back-calculate the concentration of I in the corresponding standards, and these data were pooled across experimental days to evaluate precision and accuracy.

RESULTS AND DISCUSSION

Fig. 2 shows chromatograms of blank human plasma, a control standard, and I and I.S. in human plasma. The retention times of drug and I.S. were approximately 5.5 and 7.2 min, respectively, and all peaks of interest were well resolved. Resolution (R_s) of I and I.S. was 3.7, whereas the separation factor (α) was 1.44 [5]. More than 1000 preclinical (rat and dog plasma) and clinical samples have been assayed using this method, with no interfering peaks or changes in chromatography, demonstrating high selectivity of the method.

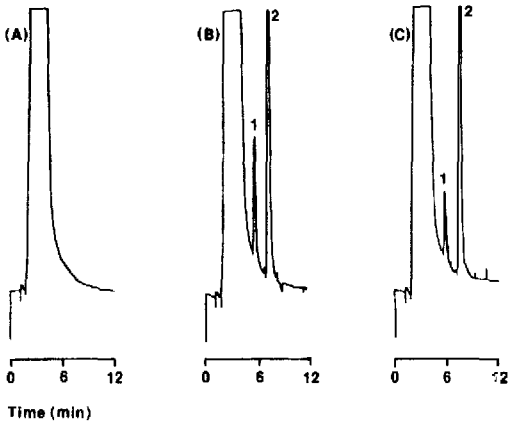

Fig. 2. Chromatograms of I in human plasma. (A) Blank plasma; (B) plasma spiked with I (10 ng/ml) and I.S.; (C) plasma control standard with I (5 ng/ml) and I.S. Detector: Kratos 773, 247 nm, output = 1 mV/mA.U. Integrator chart set at 8 mV full scale. Peaks: 1 = I; 2 = I.S.

Homogeneity of the peak of I from a dog plasma sample obtained after intravenous dosing was determined from multiple UV scans at the upslope, apex, downslope, and baseline over a range of 225–350 nm. The concentration-normalized and background-corrected spectra are shown in Fig. 3. Each of these spectra were superimposable, indicating assay specificity through peak homogeneity.

Mean extraction recoveries [with the percentages relative standard deviation (R.S.D.) in parentheses] were 82% (5.0) and 85% (2.4) from the 25 and 100 ng/ml I samples, respectively, and 82% (8.1) from I.S. samples.

At the 2 ng/ml calibration curve lower limit, the signal-to-noise ratio was 10 or greater. Under these conditions, the minimum detectable concentration of I in plasma was 1 ng/ml.

Data demonstrating linearity, precision, and accuracy of the calibration curves are given in Table I. Between-day reproducibility of the calibration curves was demonstrated by variation of the back-calculated calibration standards. R.S.D. values of the calibration standards (n = 9) ranged from 2.5 to 5.6%, with relative errors of −4.0 to 3.8%. These results indicate that the method is linear over the range 2—500 ng/ml.

Precision and accuracy were determined from analysis of control standards. Assay precision was ± 4.1%, based on R.S.D. values (n = 9) of 2.9—4.1% for control standards. The accuracy of the method was ± 3.2%, with relative errors ranging from 0.0 to 3.2%.

During development of this method, it was found that a variety of laboratory solvents commonly used for liquid—liquid extraction, or their combinations, yielded drug recoveries not greater than 50% from appropriately buffered plasma samples. Also, these extraction systems yielded poor quality and variable plasma blanks. For these reasons, liquid—solid extraction was evaluated as an alternative method for isolating I from biological samples.

A specific, selective isolation procedure was developed on Sep-Pak C_{18} cartridges after screening a variety of liquid—solid extraction devices. Pretreatment of the Sep-Pak with TBA allowed elution of I and I.S. using moderately

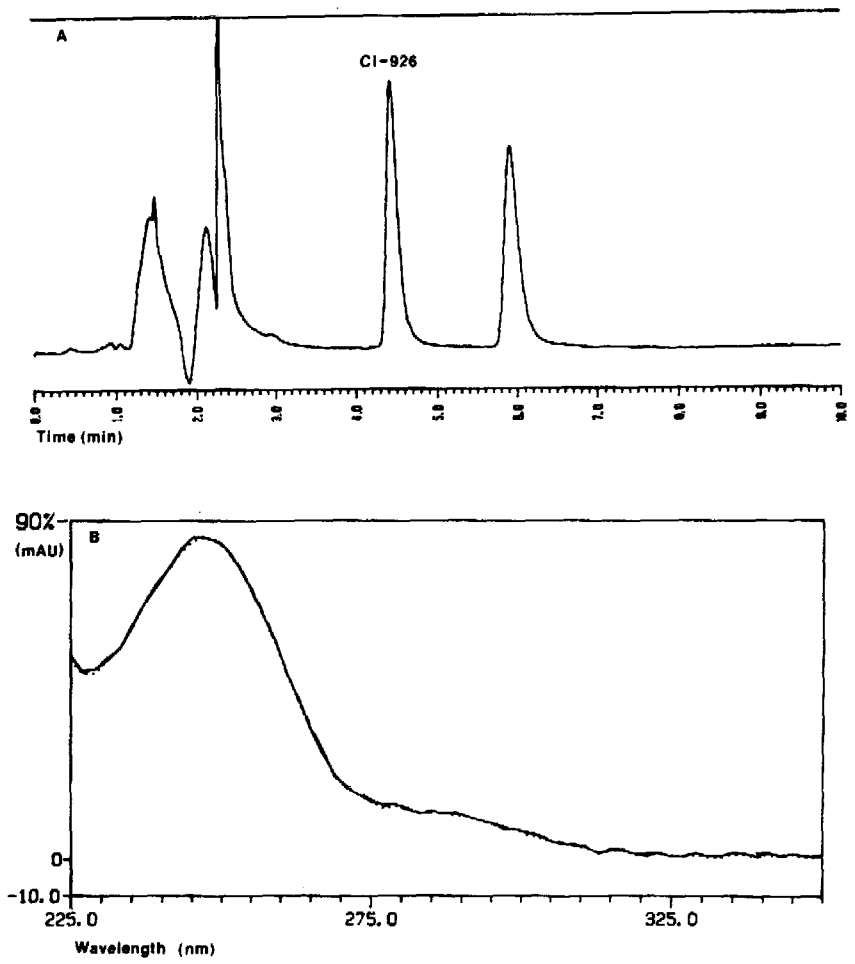

Fig. 3. Chromatogram (A) and spectra (B) from the analysis of I in dog plasma following intravenous administration. Spectra were taken from the upslope (——), apex (· · · ·), and downslope (- - - -) of the I (CI-926) peak. Detector: Hewlett-Packard 1040 scanning diode-array.

polar organic solvents. Acetonitrile, ethyl acetate, diethyl ether, chloroform, and dichloromethane all eluted I, but dichloromethane gave the best combination of clean plasma blank and recovery. Wells [6] has reported the use of TBA to mask silanophilic interaction on Sep-Pak C_{18} cartridges in the isolation of herbacides from aqueous samples. It is of interest to note that the Sep-Pak cartridges could be washed with hexane which apparently removed residual lipid and aqueous materials without eluting I or I.S.

The first column liquid chromatographic separation was achieved on an octadecylsilane column using an acetate buffer—acetonitrile mobile phase, but a combination of factors including poor peak shape, variable retention behavior, and plasma interferences led to evaluation of other stationary phases. Many combinations of mobile phases (with and without ion-pairing agents) and reversed-phase columns were evaluated. Virtually all conventional reversed-phase systems yielded less than ideal results. Poor peak shape is not uncommon

TABLE I

PRECISION AND ACCURACY RESULTS FROM A THREE-DAY TRIPLICATE STANDARD VALIDATION STUDY

Concentration of I (ng/ml)		Percentage deviation** from theoretical	Relative standard deviation (%)
Added	Found*		
Calibration standards			
2	2.0	0.0	5.6
5	4.8	−4.0	4.9
10	9.8	−2.0	3.4
50	48.3	−3.4	3.6
100	101.2	1.2	2.5
200	203.7	1.9	4.3
500	519.2	3.8	3.8
Control standards			
5	5.0	0.0	4.1
50	51.3	2.6	2.9
200	206.3	3.2	3.7

*Mean of nine values pooled from three separate calibration curves.
**Percentage deviation = (found − added)/added × 100%.

when attempting to separate amine-containing drug molecules using conventional reversed-phase chromatographic methods, and has been attributed to combined interaction of drug molecules with the bonded stationary phase and silanol groups on the unreacted surface of the silica gel support material [7, 8].

Our need to mask silanols during liquid—solid extraction indicated that silanophilic interaction could be exploited for use as the main separation mechanism. The strong interaction of the drug with unmodified silica gel in an aqueous—organic mobile phase provided a specific and selective separation. This type of interaction has been reported by Bidlingmeyer et al. [9] for a variety of lipophilic amine drugs, and has been attributed to an ion-exchange interaction between the drug and surface silanol groups.

Although drug elution patterns for these systems are similar to those of alkyl-bonded reversed-phase columns, entirely different selectivities are offered for separation of interfering plasma components when used with a variety of amine drugs and liquid—liquid extractions (unpublished observations).

In our application, the first cartridge of the analytical column does not function as a conventional guard column, but rather as an easily replaced column front. Columns have been used for as many as 1000 plasma samples by replacing the first cartridge upon increases in pressure or decreases in resolution. In general, the first cartridge required replacing every 200 to 300 samples. The matching presaturator column allowed the long-term use of an alkaline semi-aqueous mobile phase (apparent pH 8.3) without significant erosion of the microparticulate silica analytical column. In addition, our use of a 2.1 mm I.D. column yielded an approximate four-fold increase in peak height due to reduction in elution volume, yet no special equipment or flow cells were necessary [10].

The high selectivity of this method is due to the use of an octadecyl reversed-phase liquid—solid extraction with a completely different, ion-exchange-based analytical separation. Similar selectivity is possible by combining more conventional liquid—liquid extractions with separations on unmodified silica gel columns. These methods could prove useful in the analysis of many lipophilic amine drugs.

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