Analysis of Prazosin in Plasma by a Sensitive High-Performance Liquid Chromatographic–Fluorescence Method

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Abstract □ A specific high-performance liquid chromatographic–fluorescence method for the quantitative analysis of prazosin in plasma at concentrations down to 0.2 ng/ml is described. The method involves the coextraction of drug and an internal standard from alkalinized plasma followed by a simple purification step prior to evaporation and high-performance liquid chromatographic–fluorescence analysis. The method is sufficiently sensitive to allow pharmacokinetic analyses of 1-mg doses through five half-lives, with a relative standard deviation of 12%.

Keyphrases □ Prazosin—high-performance liquid chromatographic–fluorescence analysis in plasma □ High-performance liquid chromatography–fluorometry—analysis, prazosin in plasma □ Antihypertensives—prazosin, high-performance liquid chromatographic–fluorescence analysis in plasma

Prazosin, an antihypertensive agent of the quinazoline family, lowers blood pressure in humans at doses as low as 0.5 mg (1). The determination of plasma drug concentrations after such low doses required an assay with a sensitivity capable of measuring levels below 1 ng/ml.

The quantitative determination of prazosin in human plasma has been accomplished by fluorometric–wet chemistry methods involving drug extraction from alkalinized plasma with ethyl acetate followed by back-extraction into dilute acid (2). Concentrations were calculated from the fluorescence measured at specific excitation and emission wavelengths as compared to externally standardized samples. The assay is rapid but limited in sensitivity to approximately 2 ng/ml and requires quantitative or carefully controlled aliquot transfer steps.

The method described here is specific and accurate. By use of an internal standard, the need for quantitative transfer steps has been eliminated.

EXPERIMENTAL

Reagents and Materials—Prazosin [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furoyl)piperazine] (I) and the internal standard 4-(4-amino-6,7,8-trimethoxy-2-quinazolinyl)-1-piperazine carboxylic acid, 2-methallyl ester (II) were prepared as methanolic stock solutions at 1.0 mg/ml and stored in the dark at 4°; no degradation was noted for at least 3 weeks. Appropriate dilutions of these solutions were made weekly with methanol to produce working standards containing 1, 0.1, and 0.01 μg of prazosin/ml and 1 μg of the internal standard/ml. Acetonitrile[1] and ethyl acetate[1] were glass distilled, as was the water used in preparing the reagents. Stock reagents were analytical reagent quality unless specified otherwise. Glassware was treated with a solution of sulfuric acid and sodium dichromate and rinsed well with water before use.

Sample Preparation—In a 15-ml centrifuge tube, 2.0 ml of plasma was fortified with 20 ng of internal standard, made alkaline with 0.5 ml of 1 *N* KOH, and extracted with 5 ml of ethyl acetate on a vortex mixer for 30 sec. Following centrifugation, the solvent was decanted into another 15-ml centrifuge tube, 1 ml of 0.1 *N* H_2SO_4 was added, and the contents of the tube were mixed on a vortex mixer for 10 sec. After centrifugation, the organic solvent layer was aspirated and discarded; the acidic aqueous fraction was made alkaline by the addition of 0.5 ml of 1 *N* KOH and extracted with 5 ml of ethyl acetate on a vortex mixer for 30 sec.

After centrifugation, the organic solvent was recovered, transferred to another 15-ml centrifuge tube with a disposable pipet (Pasteur), and evaporated to dryness at 50° in a water bath using a nitrogen stream. The residue was reconstituted with 50 μl of the chromatographic mobile phase (below), and aliquots (1–10 μl) were assayed by high-performance liquid chromatography (HPLC)–fluorescence. Samples at this stage could be held for at least 7 days when stored in the dark at 4°, as demonstrated by the same peak height ratio of prazosin to the internal standard measured on Days 1, 3, and 8. The peak height ratios were 0.99, 0.99, and 1.01, respectively, for a concentration of 2 ng/ml of drug and 10 ng/ml of the internal standard (n = 3).

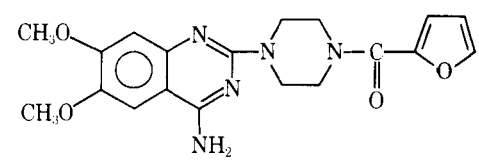
Table I—Calibration Curve from Plasma

Fortification Level, ng/ml	Number of Samples	Average PHR[a], Drug/Internal Standard
0	3	0
0.2	2	0.083
0.5	2	0.20
1.0	2	0.47
2.0	4	0.85
5.0	2	1.81
10.0	2	3.75
20.0	2	7.24

[a] PHR = peak height ratio.

HPLC—An analytical liquid chromatograph[2], equipped with a 4 × 300-mm 10-μm column[3], was fitted with a fluorescence detector[4] using low dead-volume hardware. A mobile phase of acetonitrile–water–acetic acid (50:47:3) was filtered, degassed, and used at a flow rate of 2.4 ml/min (99.12 kg/cm²). The effluent stream was monitored at an excitation wavelength of 246 nm while employing the KV 389 emission filter. The range setting was varied from 0.2 to 1.0 μamp using a time constant of 2 sec. The signal was monitored with a 10-mv strip-chart recorder using a chart speed of 0.254 cm/min. Column effluent was recycled back into the mobile phase reservoir (700 ml) and prepared weekly.

Calibration curves were constructed by determining the response from known amounts of prazosin and the internal standard added to control plasma. Electronic integration offered no advantages and did not appear to yield results as reproducible as manual measurement of peak heights. Assay linearity was demonstrated over the 0.2–20-ng/ml range (Table I); the amount of the internal standard was held constant at 20 ng/sample.



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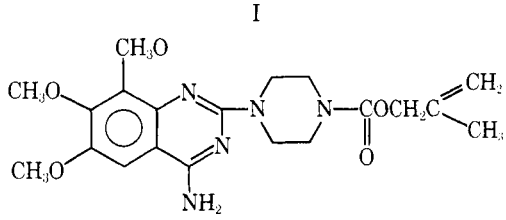

II

[1] Burdick & Jackson Laboratories, Muskegon, Mich.

[2] Spectra-Physics model 3500.

[3] The packing material was μBondapak CN; the column was Part 84042, Waters Associates.

[4] Model 970, Schoeffel Instrument Corp.

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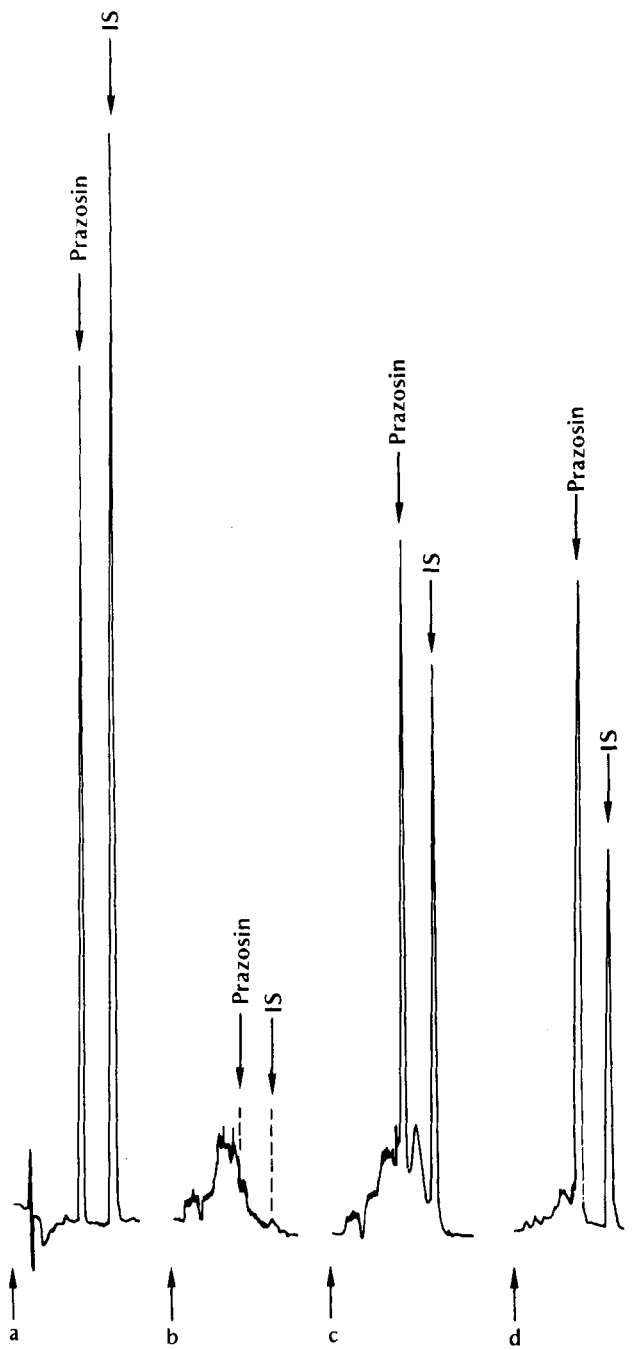

Figure 1—*Chromatograms showing the separation of drug and the internal standard (IS) (a), control plasma (b), control plasma fortified at 2 ng/ml with drug and at 10 ng/ml with the internal standard (c), and 8-hr plasma of Subject HS after a 1-mg dose with 10 ng/ml of the internal standard added (d). Retention times were prazosin, 4.2 min; and the internal standard, 6.2 min.*

For daily validation, five samples fortified at midrange (2.0 ng/ml) were processed with each group of test samples. The mean ratio of prazosin to the internal standard (IS) peak heights was determined, and the prazosin concentration in test samples was calculated from the formula:

$$\text{concentration (ng/ml)} = \frac{\text{peak height drug}}{\text{peak height IS}} \times \frac{C}{\text{mean ratio}} \quad \text{(Eq. 1)}$$

where C represents the concentration (nanograms per milliliter) of prazosin in the fortified standards.

To assess assay specificity, plasma samples from six volunteers receiving 5 mg of prazosin were pooled, made alkaline, and extracted with two volumes of ethyl acetate. The organic layer (180 ml) was recovered, extracted with 20 ml of 0.1 N H_2SO_4, and discarded. The acidic aqueous phase was then alkalinized and extracted with 40 ml of ethyl acetate. The organic layer was recovered and reduced in volume under vacuum to 0.2 ml, and 50-μl aliquots were applied to TLC plates[5].

Following development in equilibrated tanks containing ethyl acetate–diethylamine (95:5) (R_f 0.43) or chloroform–methanol–diethylamine (90:5:5) (R_f 0.63), the plates were visualized using 254-nm UV light. Only one fluorescent zone, migrating with the same R_f as prazosin, was visible on each plate. This evidence, together with the absence of additional peaks during HPLC (Fig. 1), indicated that only unchanged prazosin was being measured in the assay.

RESULTS AND DISCUSSION

Reproducibility was calculated by normalizing the observed ratios over the assay range (Table I) to 1.0 ng/ml. A relative standard deviation of 12.0% was found (n = 16). Assay recovery was determined by comparing the response from known amounts of drug and the internal standard with processed fortified plasma samples. Assay recoveries for prazosin and the internal standard were 58.6 and 50.2%, respectively.

Mean peak plasma levels of 14.9 ng/ml were reported following a 2-mg oral dose to hypertensive humans (1). In an extensive pharmacokinetic investigation with normal subjects, mean peak concentrations of 37.5 ng/ml following a 5-mg capsule were observed (3). Thus, peak concentrations of about 7.5 ng/ml after a 1-mg dose can be expected, assuming a continued linear relationship between dose and plasma levels. The 0.2-ng/ml sensitivity of the assay described here can be expected to permit the measurement of plasma prazosin levels through five half-lives following a 1-mg dose to humans.

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[5] Silica gel glass plates supplied by Brinkmann Instruments, Westbury, N.Y.