Journal of Chromatography, 172 (1979) 313–318
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,643

PRAZOSIN DETERMINATION BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY USING FLUORESCENCE DETECTION

YIN GAIL YEE, PETER C. RUBIN* and PETER MEFFIN**

Pharmacokinetics Laboratory, Division of Cardiology and the Division of Clinical Pharmacology, Department of Medicine, Stanford University Medical Center, Stanford, Calif. 94305 (U.S.A.)

(Received December 1st, 1978)

SUMMARY

A high-pressure liquid chromatographic procedure using fluorescence detection for the analysis of prazosin in whole blood and plasma is described. It employs a simple and rapid method of sample preparation. Prazosin and an internal standard are chromatographed as ion-pairs with pentane sulfonic acid. The method is sensitive and reproducible with accurate detection as low as 0.2 ng/ml in whole blood and 0.5 ng/ml in plasma with a coefficient of variation of 5.6% and 5.4%, respectively. If propranolol or quinidine is present, modifications of chromatographic conditions are used which separate prazosin from these two drugs. Prazosin is stable when frozen for a long period or refrigerated for short periods.

INTRODUCTION

Prazosin is a vasodilating agent which has gained widespread acceptance in the management of hypertension[1] and which is currently undergoing efficacy studies as an afterload reducer in heart failure[2]. Prazosin has previously been analysed fluorometrically using a xenon arc source at 340 nm[3]. The fall off in power of this source at low wavelengths produces an artificial excitation maximum at 340 nm. When prazosin is excited with a deuterium lamp, and when the emission spectrum is monitored at 390 nm, excitation at 246 nm results in a relative fluorescence which is ten times that produced by excitation at 340 nm.

Twomey and Hobbs[4] have recently published a method for the analysis of prazosin in plasma in which they employed high-pressure liquid chromatography (HPLC) and fluorescence detection at an excitation wavelength of 246 nm. Since the study of clearance of drugs with the dispositional characteristics of prazosin requires whole blood concentration data[5], we have applied HPLC–fluorescence detection to the analysis of prazosin in whole blood. The method we describe uses a simple and rapid two step extraction procedure with different chromatographic conditions than

* To whom correspondence should be addressed.

** Present address: Department of Clinical Pharmacology, Flinders University Medical School, Adelaide, South Australia (Australia).

previously described, leading to more rapid separation but with no loss of sensitivity. In addition, we describe studies on the stability of prazosin under storage and the potential interference of other cardiovascular drugs.

EXPERIMENTAL

Reagents and materials

Prazosin 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2 furanylcarbonyl)piperazine as the hydrochloride salt, and the internal standard, the 2-methallyl ester of 4-(4-amino-6,7,8-trimethoxy-2-quinazolinyl)-1-piperazine carboxylic acid (Fig. 1), were supplied by Pfizer (Groton, Conn., U.S.A.). Stock solutions were prepared in methanol from which dilutions were made in water to produce working standards. The working standards were stored at 4° for approximately a month with no detectable decomposition. Glass distilled water was used for standard solutions, chromatography and assay preparation. The methanol used in stock solution preparation and chromatography was "distilled in glass" quality and purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Pentane sodium sulfate was purchased from Eastman-Kodak (Rochester, N. Y. U.S.A.). All other solvents and reagents were reagent grade quality.

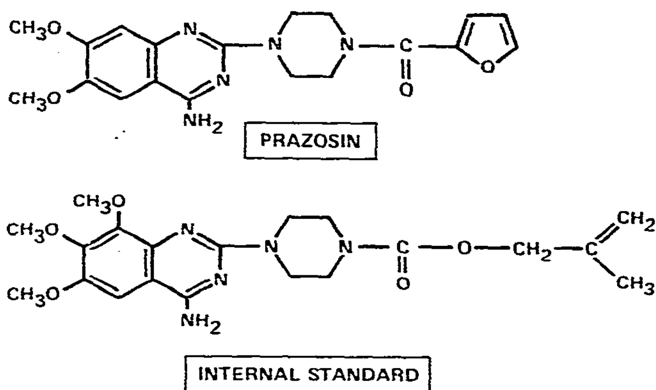

Fig. 1. Structures of prazosin and internal standard.

Sample preparation

A schematic outline of this method is shown in Fig. 2. Whole blood or plasma, 0.1–1.0 ml, was added to an 8-ml capacity culture tube fitted with a PTFE-lined screw cap and containing 54 ng of the internal standard added to 100 μl of water. Water was added to samples with less than 1.0 ml, so that all tubes had an equal volume of aqueous phase. The blood was alkalinized with 200μl of 2 N sodium hydroxide and extracted immediately with 5 ml diethyl ether to prevent the formation of solid aggregates. Samples were mixed on a Labquake for 10 min and then centrifuged for 10 min. The aqueous phase was frozen by placing the tube in an acetone–dry ice bath and the organic phase decanted into an 8-ml tube with an elongated cone at its base, of approximately 30-μl capacity, containing 20 μl of 0.1 N sulfuric acid. The sample was extracted with a Vortex mixer for 1 min, the tubes chilled in the refrigerator for

10 min and then centrifuged for 5 min. All or part of the dilute sulfuric acid, sampled through the diethyl ether with a 25-μl syringe, was injected into the high-pressure liquid chromatograph.

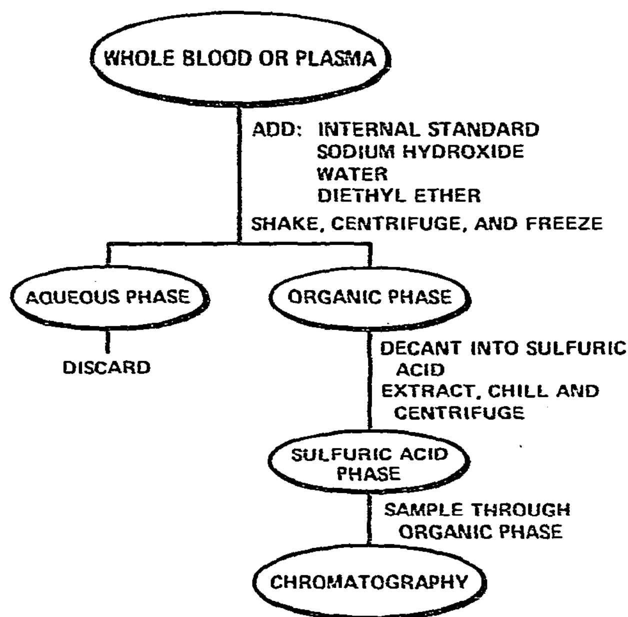

Fig. 2. Schematic outline of the sample preparation used in the analysis of prazosin in whole blood and plasma.

Chromatography

A Varian model 8500 dual pump gradient elution high-pressure liquid chromatograph fitted with a Varian Micro-Pak MCH-10 (monomeric C_{18} bonded) reversed-phase column (25 cm × 2.0 mm I.D.) was used for the analysis. One pump contained a 0.01 *M* solution of pentane sodium sulfate in water adjusted to pH 3.4 with glacial acetic acid (solvent A). The other pump contained the same concentrations of pentane sodium sulfate and acetic acid as solvent A in methanol (solvent B). Both solvents were filtered before use. An isocratic mixture of 49% solvent B and 51% solvent A was used with daily minor adjustments in solvent composition (1–2%) to maintain optimum baseline separation of prazosin and the internal standard. The flow-rate of the solvent mixture was 40 ml/h, with a column input pressure of 150 atm (2300 p.s.i.). The column was insulated with sponge rubber in order to minimize baseline noise. A Varian Fluorichrom with a deuterium lamp and Baird Atomic 20-nm bandpass filters, 253.1 nm for excitation and 390 nm for emission was used. A 0.5-μm porosity stainless-steel frit was placed on the efferent side of the detector to maintain the detector pressure thereby preventing formation of bubbles. A Varian A-25 dual pen recorder was employed with one pen set at 1 mV full scale deflection and the other varied between 2 mV–50 mV depending on the expected concentration of the sample.

Calibration and accuracy

The assay was calibrated by adding known amounts of prazosin (0.2 ng–50 ng)

and internal standard (54 ng) to 1 ml of whole blood or plasma which was then analysed. The peak height ratio (PHR) of prazosin to the internal standard was plotted *versus* the amount of prazosin added. To determine the accuracy and precision of each set of unknown samples a calibration curve consisting of 0.2, 0.5, 1, 2, 5, 10, 20 and 50 ng of prazosin was assayed along with the unknown samples. The PHR of the standard samples were divided by the amount of the prazosin added to derive the normalized PHR for 1 ng/ml. The mean normalized PHR was used to calculate the amount of prazosin in the unknown samples and the coefficient of variation provides an estimate of the accuracy of the method over the range of standard samples.

The reproducibility of the method was investigated by analysing five replicate samples in whole blood and ten replicate samples in plasma of 2 ng and 20 ng concentrations of prazosin. The effect of variable sample size was studied using 0.2–2.0 ml of whole blood or plasma without the addition of water, keeping constant the volume of internal standard solution, sodium hydroxide, and diethyl ether. The recovery of prazosin was determined by comparing the peak heights of extracted known concentrations of prazosin injected directly into the chromatograph.

The stability of prazosin in heparinized whole blood was investigated by assaying samples after they had been frozen at −20° for 6 months, refrigerated for up to 5 days or left at room temperature for 1 h, 2 h, 3 h, or 5 h. The internal standard was added at the time of analysis. Concentrations in each stored set were calculated from the normalized PHR of a freshly prepared calibration curve analysed on the same day.

The assay specificity was assessed by taking various cardiovascular drugs which might be co-administered with prazosin, through the extraction and chromatography and comparing the retention times to those of prazosin and the internal standard.

RESULTS AND DISCUSSION

Under the chromatographic conditions described above, the retention times of prazosin and the internal standard were 3.0 and 4.5 min, respectively. Fig. 3A shows the chromatogram of an analysis of 1 ml of whole blood containing 5 ng of prazosin and 54 ng of internal standard. Fig. 3B and C show chromatograms of 1 ml of control whole blood and plasma, respectively, taken through the analysis. No peaks corresponding to the peaks shown in Fig. 3A have been found in assaying control samples of whole blood and plasma from approximately 30 subjects.

A typical calibration curve from whole blood is linear in the range of 0.2 ng to 50 ng/ml of prazosin with a regression coefficient of 0.9997 for the line $y = 0.03079 + 0.1070\,x$, where y is the peak height ratio and x the concentration in ng/ml. The average coefficient of variation for the normalized PHR over this range is 5.6%. A plasma calibration curve in the range from 0.5 ng to 50 ng/ml of prazosin has a coefficient of variation of 5.4% with a linear regression coefficient of 0.9256 for line $y = 0.2643 + 0.1516\,x$. Limits of detection, arbitrarily defined as 3 times baseline noise, are 0.1 ng/ml in whole blood and 0.2 ng/ml in plasma. A limiting factor in the lesser sensitivity in plasma appears to be a fluorescing peak seen in plasma but not in whole blood (Fig. 3). This interfering peak was also seen under the extraction and chromatographic conditions employed in the previously described HPLC assay of prazosin[4].

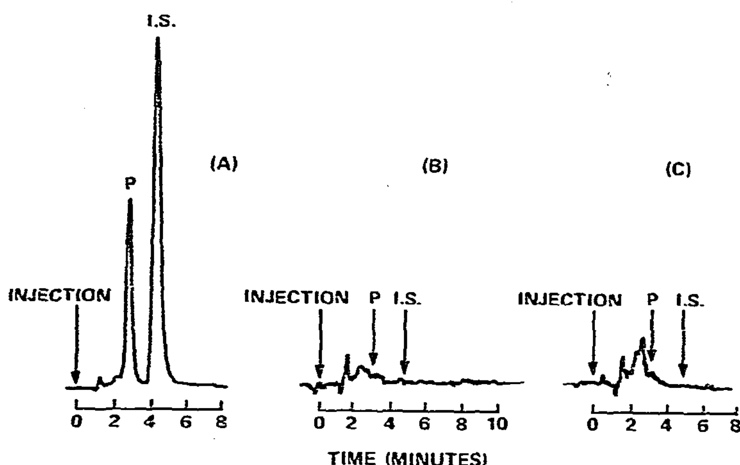

Fig. 3. A: Chromatogram of extracted whole blood containing 5 ng of prazosin (P, 3.0 min) and 54 ng of internal standard (I.S., 4.5 min) with attenuation at 5 mV full scale deflection. B and C: Chromatograms of extracted control whole blood and plasma, respectively. No peaks are seen corresponding to prazosin (P) and internal standard (I.S.), with attenuation at 2 mV full scale deflection.

In order that peak height ratios should fall within the range of the calibration curve it is often necessary to estimate the result of the unknown samples and adjust sample size accordingly. It was found that a constant 1:5 ratio of aqueous to organic phase was needed to maintain a reproducible PHR. Reproducibility studies of 2 ng and 20 ng per sample gave a coefficient of variation of 4.4% and 3.6% in whole blood and 6.7% and 2.4% in plasma, respectively.

After storage at room temperature there was a tendency for the prazosin value to decrease, but this was no more marked at 5 h than at 1 h and was within the range of the coefficient of variation. Refrigeration tended to produce somewhat higher values, but again within the coefficient of variation. Results of fresh samples and samples frozen for six months were not statistically different. Because it is sometimes convenient to extract samples on one day and chromatograph them the next, the stability of prazosin extracted into the acid phase was also studied. The mean PHR on the second day was 97% of that found on the first.

The specificity of the assay is shown in Table I with the retention times of various cardiovascular drugs as compared to that of prazosin and the internal standard. Furosemide, procainamide, and *n*-acetylprocainamide do not interfere with the chromatography of either prazosin or the internal standard. Bumetanide is not extracted by the method described for prazosin. Quinidine and propranolol can both be separated from prazosin by slowing the chromatographic retention times. For the separation of quinidine and prazosin, a change to 40% solvent B–60% solvent A alters the retention times to 3.9 min and 6.0 min, respectively. For propranolol and prazosin a necessary change to 45% solvent B–55% solvent A gives baseline separation with retention times of 2.9 and 4.0 min, respectively.

The method described here for the quantitative determination of prazosin has the advantage of being a simple and rapid two-step extraction procedure for both whole blood and plasma with no loss of sensitivity. Using the techniques described 30–40 samples can easily be extracted and chromatographed in a day. The use of

TABLE I

THE RETENTION TIMES OF OTHER CARDIOVASCULAR DRUGS UNDER THE CHROMATOGRAPHIC CONDITIONS DESCRIBED

Drug	*Retention time (min)*
Prazosin	3.0
Internal Standard	4.5
Acebutolol	no peak
Atenolol	no peak
Bumetanide	3.1*
Digoxin	no peak
Disopyramide	no peak
Furosemide	1.6
Hydralazine	no peak
Methyldopa	no peak
Metoprolol	no peak
Mexiletine	no peak
n-Acetyl procainamide	2.0
Procainamide	1.8
Propranolol	2.6**
Quinidine	2.7**

* Not extracted by procedure.
** Can be separated by a modification of conditions (see text).

whole blood minimizes the sample size required and allows the valid analysis of blood clearance. Previous methods have not investigated possible interference from other cardiovascular drugs and it is of considerable interest, therefore, that propranolol and quinidine have been found to possess chromatographic and fluorescence characteristics very similar to those of prazosin.

This procedure is currently being routinely used in pharmacokinetic studies with prazosin.

ACKNOWLEDGEMENTS

The prazosin and internal standard were kindly supplied by Donald C. Hobbs, Ph.D., Pfizer Central Research.

We wish to thank Sandra Harapat for early assistance in the development of this method and Linda Halloran for preparation of the manuscript.

Dr. Rubin is a Research Fellow of the American Heart Association.

This work was supported by NASA-NGS 2294, NIH 1-R01-HL-21221 and GM 22209.

REFERENCES

1 R. N. Brogden, R. C. Heel, T. M. Speight and G. S. Avery, *Drugs*, 14 (1977) 164.
2 N. A. Awan, R. R. Miller, A. N. De Maria, K. S. Maxwell, A. Neumann and D. T. Maso·· *Circulation*, 56 (1967) 346.
3 A. J. Wood, P. Bolli and F. O. Simpson, *Brit. J. Clin. Pharmacal.*, 3 (1976) 199.
4 T. M. Twomey and D. C. Hobbs, *J. Pharm. Sci.*, 67 (1978) 1468.
5 M. Rowland, L. Z. Benet and G. G. Graham, *J, Pharmacokinet. Biopharm.*, 1 (1973) 123.