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ATENOLOL DETERMINATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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SUMMARY

A high-performance liquid chromatographic procedure using a fluorescence detector for the analysis of atenolol in plasma and whole blood is described. It employs a simple and rapid method of preparation. Atenolol and metoprolol as the internal standard are chromatographed as ion pairs with heptanesulfonic acid. The method is sensitive and reproducible with accurate detection at concentrations as low as 2 ng/ml in whole blood and plasma, and a coefficient of variation of 4.7% over the range 2 ng/ml to 1000 ng/ml.

INTRODUCTION

Atenolol is a new beta-adrenergic receptor antagonist. The therapeutic advantages of this drug include selectivity for beta-1 receptors[1] and efficacy in treating hypertension when administered once daily[2]. Three methods for the analysis of atenolol have previously been described but none has combined sensitivity, specificity, ease of performance and general applicability to clinical situations. The spectrofluorometeric method is simple but its limit of detection is 50 ng/ml and it lacks specificity[3]. The gas chromatography determination of atenolol is specific and sensitive to 10 ng/ml but requires a lengthy derivatization step[4]. The third method employing high-performance liquid chromatography (HPLC) and fluorescence detection[5] has three major difficulties: column life is very short; the internal standard is procainamide, a drug which might be administered in combination with atenolol; and the limit of sensitivity is 20 ng/ml, which makes it less useful for single-dose kinetic studies.

We have developed a simple HPLC assay which overcomes the problems outlined above. The method employs paired-ion chromatography. Metoprolol, a beta blocker unlikely to be prescribed with atenolol, is the internal standard. The procedure accurately measures atenolol concentrations as low as 2 ng/ml in whole blood or plasma.

EXPERIMENTAL

Reagents and materials

Atenolol [4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetamide] supplied by Stuart Pharmaceuticals (Wilmington, Del., U.S.A.) and metoprolol (Astra, Great Britain) (see Fig. 1) were used for the analysis.

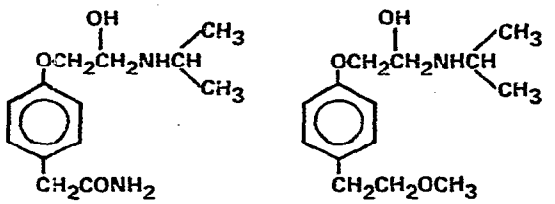

Fig. 1. Structures of atenolol and metoprolol.

Stock solutions of atenolol and metoprolol were prepared in water and appropriate dilutions of the atenolol stock solution were made to produce working standards. Stock and standard solutions were stored at 4° with no detectable decomposition of atenolol for over a month and metoprolol for over 8 months.

The water used for standard solutions, chromatography and assay preparation was glass distilled. The methanol used in chromatography was "distilled in glass" quality, purchased from Burdick & Jackson (Muskegon, Mich., U.S.A.). Heptane sodium sulphate was purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.). All other solvents and reagents were reagent grade quality.

Sample preparation

A schematic outline of this method is shown in Fig. 2. Plasma or whole blood, 0.1–1.0 ml, is added to an 8-ml capacity culture tube (fitted with a teflon-lined screw cap) containing 200 ng of metoprolol in 100 μl of water. For sample volumes less than 1.0 ml, water is added to bring all tubes to an equal volume of aqueous phase. Blood or plasma is alkalinized with 200 μl of 2 *N* NaOH and extracted with 55% cyclohexane–1-butanol (5 ml). Samples are mixed on a Labquake® shaker for 10 min and then centrifuged for 10 min. The organic phase is transferred with pasteur pipets to a second tube with an elongated cone (capacity *ca.* 50 μl) at its base. This cone contains 40 μl of 0.1 *N* H_2SO_4. The sample is extracted with a Vortex® mixer for 1 min, placed in the refrigerator for 10 min and then centrifuged for 5 min. All or part of the dilute sulfuric acid, sampled through the organic solvent with a 50-μl syringe, is injected into the chromatograph.

Chromatography

A Varian 8500 dual pump gradient elution high-performance liquid chromatograph fitted with a Varian MicroPak MCH-10 (monomeric C_{18} bonded phase) reversed-phase column (25 cm × 2.0 mm I.D.) was used for the analysis. One pump contained 0.01 *M* heptanesulfonic acid in water adjusted to pH 3.4 with glacial acetic acid (solvent A). The other pump contained the same concentration of heptane sodium

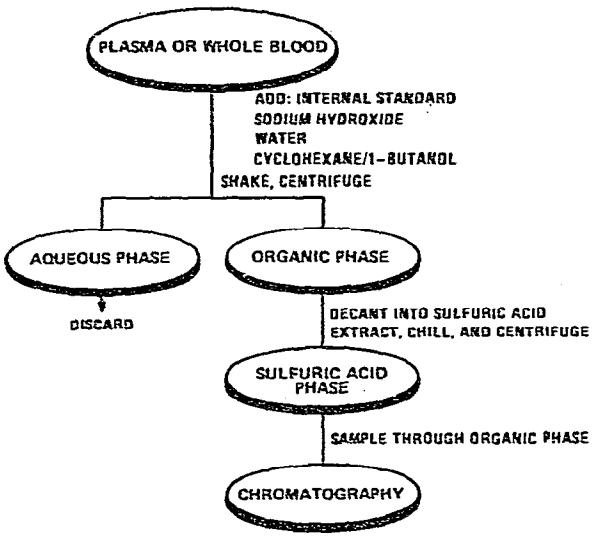

Fig. 2. Schematic outline of the sample preparation used in the analysis of atenolol in whole blood or plasma.

sulfate and acetic acid as solvent A but dissolved in methanol (solvent B). Both solvents were filtered before use. An isocratic mixture of 28% solvent B and 72% solvent A was used with occasional minor adjustments in solvent composition (1–2%) to attain a baseline separation between atenolol and an unknown peak seen both in plasma and blood (Fig. 3). The flow-rate of the solvent mixture was 40 ml/h, with a column input pressure of 130 atm (2000 p.s.i.). The column was insulated with sponge rubber in order to minimize baseline noise. The detector used was a Varian Fluorichrom® fitted with two Baird-Atomic 30-nm band pass filters, 212 nm for excitation and 326.5 nm for emission. A 0.5-μm porosity stainless-steel frit was placed on the efferent side of the detector to maintain detector pressure, thereby preventing the formation of air bubbles in the flow-cell. A Varian A-25 dual pen recorder was employed with one pen set at 1-mV full-scale deflection and the other set at 2–20 mV depending on the expected concentration of the sample.

Calibration and accuracy

The method was calibrated by adding known amounts of atenolol (2–1000 ng) and metoprolol (200 ng) to 1 ml of plasma or whole blood, then taking these standards through the assay procedure. The peak height ratio (PHR) of atenolol to the internal standard was plotted versus the amount of atenolol added. When analysing unknown samples, a calibration curve consisting of 2, 5, 10, 50, 100, 500, and 1000 ng of atenolol is assayed with the unknowns. The PHR of the standard samples were divided by the amount of atenolol added to derive the normalized PHR. The mean normalized PHR is used to calculate the amount of atenolol in 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 taking six replicate samples of 50 ng of atenolol through the procedure. The effect of variable sample size was studied using 0.1–2.0 ml of plasma without the addition of water, keeping con-

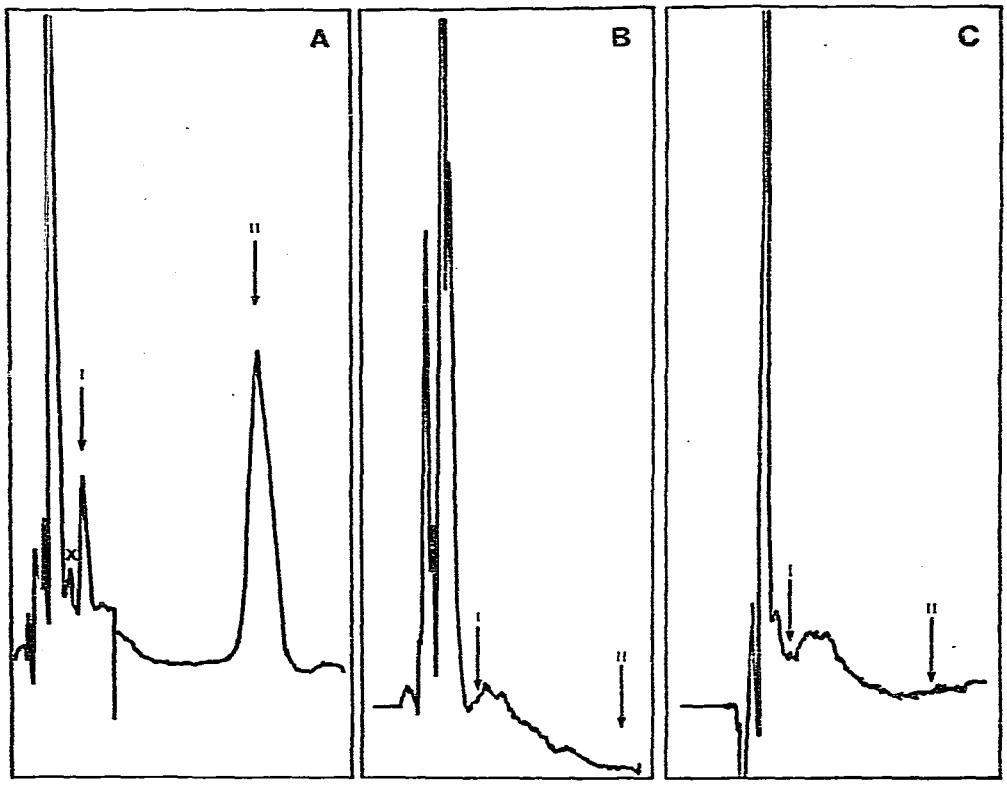

Fig. 3 (A) Chromatogram of extracted plasma containing 10 ng of atenolol (peak I, 3.5 min) and 200 ng of metoprolol (peak II, 10.8 min). Peak II attenuation 2 × peak I. Peak X is unidentified, but is seen in all samples including blank. (B) and (C) Chromatograms of extracted blank plasma and blood, respectively. No peaks are seen corresponding to atenolol (peak I) or metoprolol (peak II).

stant the volume of the internal standard, NaOH and the cyclohexane–1-butanol. Recovery of drug through the extraction procedure was determined by comparing the peak height of an extracted known amount of atenolol with the peak height of an unextracted known amount injected directly into the chromatograph.

The stability of atenolol in plasma was investigated by assaying standard samples after they had been frozen at −20° for 2 months or left at room temperature for 1 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 PHRs of a freshly prepared calibration curve analyzed on the same day.

The specificity of the assay was determined by carrying various cardiovascular drugs through the extraction and chromatography and comparing retention times to those of atenolol and the internal standard.

RESULTS AND DISCUSSION

Under the chromatographic conditions described above the retention times of atenolol and metoprolol are 3.5 and 10.8 min, respectively. Fig. 3A shows a chro-

matogram of 1 ml of plasma containing 10 ng of atenolol and 200 ng of metoprolol. Figs. 3B and C show chromatograms of 1 ml of control plasma and 1 ml of control whole blood, respectively, taken through the analysis. No peaks corresponding to the drug or internal standard retention times have been found in control samples.

A typical calibration curve from plasma is linear in the range 2–1000 ng with a regression coefficient of 0.999 for the line $y = 0.01586x + 0.07474$ where y is the peak height ratio and x the concentration in ng/ml. The average coefficient of variation for the normalized peak height ratios over this range is. 4.7%. Similarly, a whole blood calibration curve from 5 to 1000 ng has a coefficient of variation of 4.7% with a linear regression coefficient of 0.9999 for line $y = 0.01581x + 0.00977$. The limits of detection, arbitrarily defined as 5 times baseline noise, are 1 ng/ml for both plasma and whole blood.

To obtain peak height ratios which fall within the calibration curve it is often necessary to analyze samples of varying volumes. It was found that a constant ratio between the aqueous and organic phases is needed to maintain reproducible PHRs. Reproducibility studies of 50 ng and 500 ng of atenolol gave a coefficient of variation of 5.42% and 2.99%, respectively. The recovery of atenolol through the procedure is *ca.* 50% from both plasma and blood.

The accuracy of the method is dependent on maintaining a constant 55:45 ratio of cyclohexane to 1-butanol. Cyclohexane (b.p. 81.4°) and 1-butanol (b.p. 117.7°) form a 9:1 azeotrope with a b.p. of 79.8°. A mixture of cyclohexane and 1-butanol will vaporize, with cyclohexane comprising 90% of the vapor. When vaporization of the azeotrope occurs, the composition of the extraction mixture changes, and it contains a greater percentage of 1-butanol. A 5% decrease of cyclohexane, resulting in an extraction mixture of 50% cyclohexane and 50% 1-butanol decreases the extraction efficiency for atenolol by only 4%, whereas the extraction efficiency of metoprolol decreases by 66%. Therefore, to maintain a consistent normalized PHR it is necessary to prepare the 55:45 cyclohexane–1-butanol mixture daily.

Since it may be convenient to extract samples on one day and chromatograph them on the next day, the stability of atenolol extracted into the acid phase was studied. A calibration curve was extracted into acid, stored at 4° overnight, and then chromatographed. No significant difference in the normalized PHR of acid-stored samples and samples extracted and chromatographed on the same day was observed. When standard samples were analyzed after being thawed for up to 5 h at room temperature or stored at 4° for 24 h there was no trend in the PHRs. Plasma samples frozen at −20° for 2 months showed an apparent decrease in atenolol concentration of 40% over that period. This indicates that inaccurate results may be obtained if samples undergo prolonged storage.

The specificity of the assay is shown in Table I, which lists the retention times for various drugs including atenolol and the internal standard. Procainamide, N-acetylprocainamide and methyldopa did not interfere with atenolol or internal standard peaks. Only mexiletine shows potential interference with the analytical method since it has a similar retention time as the internal standard and is extracted from plasma by the procedure used.

The method described here for the quantitative determination of atenolol in plasma and whole blood has several advantages over the previously published methods. It employs a simple and rapid two-step extraction method, allowing 20–25

TABLE I

RETENTION TIMES OF CARDIOVASCULAR DRUGS UNDER THE CHROMATOGRAPHIC CONDITIONS DESCRIBED

Drug	*Retention time (min)*
Atenolol	3.5
Metoprolol	10.8
Prazosin	No peak
Furosemide	No peak
Bumetanide	No peak
Quinidine	No peak
Procainamide	5.84 (see text)
N-Acetylprocainamide	8.72
Mexiletine	10.8 (see text)
Disopyramide	No peak
Hydrallazine	No peak
Methyldopa	1.44

samples to be analyzed per day. Another beta blocker, unlikely to be used in combination with atenolol, is used as the internal standard. Under the chromatographic conditions described the expensive reverse phase column appears to be considerably more stable than has been observed with the previous HPLC method. The sensitivity is at least 10 times greater than that of previously described methods. The use of whole blood minimizes the sample sizes required. This procedure is currently being used routinely to monitor atenolol levels in clinical studies with this drug at Stanford.

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