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RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF ATENOLOL IN PLASMA USING UV DETECTION

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SUMMARY

A rapid, selective and reproducible high-performance liquid chromatographic method has been developed for the measurement of the β-adrenoceptor blocking drug atenolol in small (400 μl) volumes of plasma. Following solid phase sample preparation using Bond-Elut™ mini-columns the compound is separated by high-performance column liquid chromatography on a microparticulate (6 μm) cyano column using acetonitrile—ammonium dihydrogen phosphate (4:96) containing triethylamine (0.25%, v/v) as the mobile phase, and the absorption of the column effluent is monitored at 224 nm. The practical limit of quantitation, based upon an assay volume of 400 μl, is 25 ng/ml for atenolol. The average coefficient of variation is 3.1%.

INTRODUCTION

Atenolol, 4-(2-hydroxy-3-isopropylaminopropoxy)-phenylacetamide, is a selective β_1-adrenoceptor antagonist recently approved in the United States for the treatment of hypertension [1, 2]. Studies in animals and humans suggest that this drug is free of intrinsic sympathomimetic activity and is devoid of significant membrane-stabilizing activity [3—6].

Previous procedures for the determination of atenolol in plasma and urine employed gas—liquid chromatography (GLC) with electron-capture detection [7, 8], or high-performance liquid chromatography (HPLC) with spectrophotofluorimetry [9—12] or spectrophotometry [13]. The GLC determination of atenolol is specific and sensitive to 10 ng/ml, but is relatively complex and requires lengthy prederivatization steps. The spectrophotofluorimetric method is relatively sensitive but is of questionable specificity and employs an elaborate extraction procedure with internal standards procainamide or metoprolol, drugs which might be administered in combination with atenolol.

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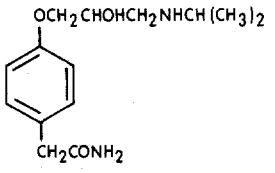
The spectrophotometric method lacks sensitivity at the wavelength chosen requiring a large volume of plasma for the assay [13].

The procedure reported here for the measurement of plasma atenolol concentrations is based upon the principle of rapid sample preparation with Bond-Elut™ columns of a relatively small plasma volume (400 μl), followed by the separative capability of HPLC and the sensitivity of UV detection.

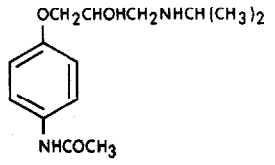
EXPERIMENTAL

Materials and reagents

Atenolol and the internal standard practolol (Fig. 1) were supplied by Stuart Pharmaceuticals (Wilmington, DE, U.S.A.) and by ICI Pharmaceuticals (Macclesfield, Great Britain), respectively. Ammonium dihydrogen phosphate and triethylamine were purchased from Aldrich Chemicals (Milwaukee, WI, U.S.A.). Acetonitrile, acetone and methanol were of HPLC grade and obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Bond-Elut™ CN columns (column capacity 1 ml) and Vac-Elut manifold were purchased from Analytichem International (Harbor City, CA, U.S.A.).



ATENOLOL



PRACTOLOL

Fig. 1. Structures of atenolol and practolol.

High-performance column liquid chromatography

The solvent delivery system is a constant-flow reciprocating Constametric III pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The analytical column is a prepacked (25 cm × 4.6 mm I.D.) stainless-steel column containing Zorbax™ CN (6 μm) polar bonded-phase packing used in the reversed-phase mode (DuPont, Wilmington, DE, U.S.A.). A six-port rotary valve injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) with a 50-μl sample loop is used for sample injection. A cyano guard column cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.) is used between the injector and the analytical column which effectively minimizes the accumulation of particulate matter on the analytical column. The mobile phase is acetonitrile—0.0125 *M* ammonium dihydrogen phosphate—triethylamine (4:96:0.25, v/v), the pH being adjusted to 5.5 with 1.0 *M* phosphoric acid. The solvent flow-rate is 1.5 ml/

min with a column inlet pressure of 103.4 bars. The eluate is monitored continuously for absorbance at 224 nm using a variable-wavelength Spectromonitor III (Laboratory Data Control) and the detector output is displayed on a Linear Instruments Model 858 dual-pen recorder (Irvine, CA, U.S.A.).

Bond Elut™ clean-up procedure

Sample preparation is done with Bond-Elut™ columns containing a sorbent which has been modified by covalently bonding cyanopropyl functional groups to the surface. The Bond-Elut column is positioned in the luer fittings in the Vac-Elut cover. Ten columns can be used at a time. A vacuum of 25—50 cm Hg is applied to the manifold to elute the different washes. The column is activated by passing 2 ml of methanol from a squeeze bottle followed by 2 ml of distilled water. The vacuum is turned off as soon as the water has run through to prevent the tubes from drying out. A 400-μl aliquot of patient's plasma containing the internal standard practolol in a concentration of 500 ng/ml is added to the column. For the calibration curve, pooled plasma containing the appropriate concentration of atenolol and the same concentration of practolol is used. The vacuum is turned on and the sample is drawn into the column. Each column is then filled twice with distilled water from a squeeze bottle, allowing columns to empty between fillings. This is followed by one wash with acetone. Following the last wash the vacuum is maintained until the column is dry. The cover of the manifold is then removed and the stainless-steel needles of the Vac-Elut cover are wiped with a tissue to remove drops of washing solution. Appropriately labelled collection tubes are positioned under each column. A 200-μl aliquot of eluting solvent (10 m*M* acetic acid, 50 m*M* triethylamine in methanol) is added to the column, and the vacuum is renewed. The suction is continued while two more 200-μl aliquots of the eluting solvent are added. The vacuum is turned off and combined eluents are collected. The combined eluents are evaporated under nitrogen, suspended in 80 μl of mobile phase and 50 μl are injected onto the analytical column.

Instrument calibration

Standard solutions containing atenolol at concentrations of 25, 50, 100, 250, 500, 750 and 1000 ng/ml were prepared in heparinised drug-free pooled plasma by dilution of a 1 mg/ml solution of this compound in methanol. The plasma and methanolic solutions were stable for at least one month, if stored at −20°C and in the absence of light. The amounts of drug in the unknown samples were calculated from their peak heights relative to the internal standard by linear regression using the reciprocal of the concentration ($1/C$) as the weighting factor [14]. To quantitate the lower concentrations with better relative accuracy it was necessary to employ $1/C$ as the weighting factor rather than unit weighting which is generally used in linear regression analysis. As an alternative to weighted linear regression two standard curves could be used, one covering the lower range (25—500 ng/ml), another covering the higher range (500—1000 ng/ml) of concentrations. The advantage in choosing the former method lies in its convenience, and the simplicity of having one equation to define the entire concentration range.

The reproducibility of the method was investigated by taking the coefficient of variation of five curves over the entire range of 25—1000 ng/ml. This was calculated from the standard deviation of the values divided by the mean peak height ratio (atenolol/internal standard) for each concentration. Standard curves based upon peak height of drug to the peak height of the internal standard were linearly related to concentration with the correlation coefficient being consistently greater than 0.998.

RESULTS AND DISCUSSION

Choice of separation

GLC with electron-capture detection [7, 8] was previously employed for determination of atenolol in plasma. Although the GLC methods are highly selective and sensitive they require lengthy derivatization steps.

Due to the low volatility of atenolol, HPLC was evaluated as an alternative method of resolving the compound prior to detection. The retention behavior

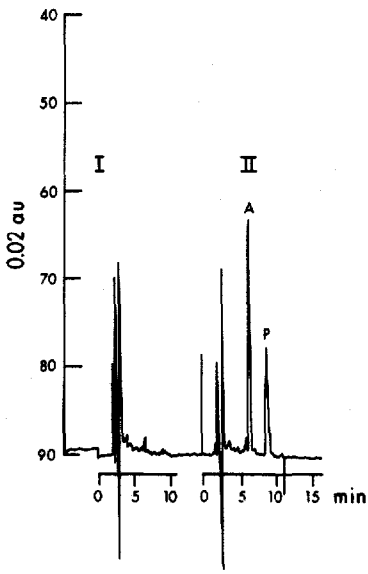


Fig. 2. High-performance liquid chromatograms of extracts from plasma. (I) Control plasma; (II) plasma containing 250 ng/ml of atenolol (A) and internal standard, practolol (P).

of atenolol was compared on three different column sorbents, namely CN (normal- and reversed-phase), C_{18} (reversed-phase) and silica gel absorption columns. Of the microparticulate columns tested the Zorbax CN column (in the reversed-phase mode) exhibited the greatest separation selectivity. The capacity factor (k') values of atenolol and the internal standard practolol were 2.0 and 3.3, respectively. The chromatographic separation using this method was excellent as can be seen from the representive chromatograms of these compounds (Fig. 2). Furthermore the chromatographic time is less than 10 min.

Choice of HPLC detection mechanism

The two most widely used forms of sample detection of HPLC effluents are UV absorption and fluorimetric methods. Electrochemical detection techniques are now being applied to electrochemically active drugs. UV, fluorimetric and electrochemical scans were obtained on atenolol (Fig. 3). Spectral scans were performed by preparing a 250 μM solution of atenolol in the mobile phase. In the case of the UV scan a Beckman Spectrophotometer UV 5230 (Beckman Instruments, Irvine, CA, U.S.A.) was used at a scan speed of 1 nm/sec and chart speed of 10 nm/in. Fluorimetric scans were performed on an Amicon SPF-500 (American Instrument Company, Silverspring, MD, U.S.A.) at a scan speed of 250 nm/sec. The electrochemical scan was performed using a CV-1B cyclic voltammetric apparatus (Bioanalytical Systems, Lafayette, IN, U.S.A.) using a glassy carbon electrode and Ag/AgCl reference electrode at a scan rate of 250 nV/sec. In all situations a blank spectrum on the mobile phase was obtained.

The molar absorptivity at 224 nm (Fig. 3A) is sufficiently intense to permit atenolol detection in a small volume of plasma (400 μl). The drug was found to have intrinsic fluorescence (Fig. 3B) and previous methods have employed spectrophotofluorimetric methods to determine atenolol in plasma [9—11]. Our method, with a smaller sample volume, was able to obtain comparable sensitivity after a much simpler and more specific extraction procedure with UV detection. Since UV detection is the most widely used detection system our method can be widely applied without the need for a spectrophotofluorimeter. Atenolol was found to have no electrochemical activity, and this method of detection is therefore not applicable.

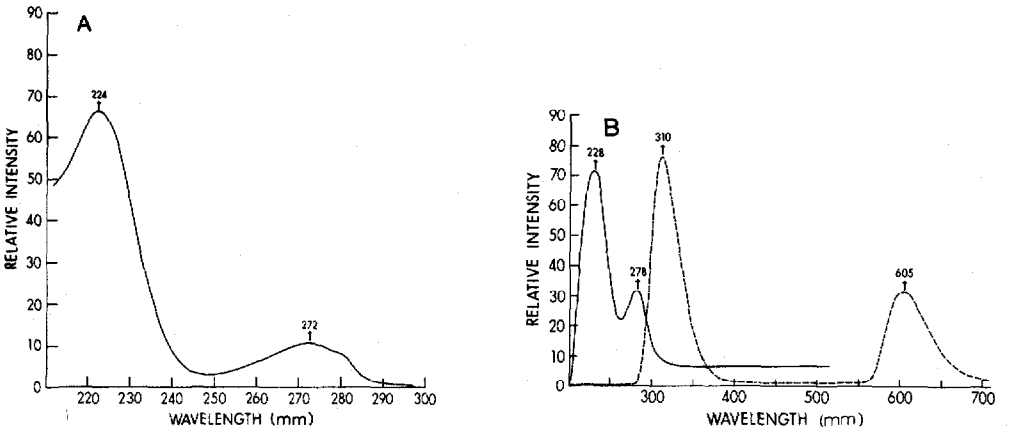


Fig. 3. Spectral scans of atenolol (for conditions see text). (A) Absorption spectrum of atenolol (UV); and (B) excitation (——) and emission (— — —) spectra of atenolol (fluorescence).

Effect of the solvent strength, pH and triethylamine content of the mobile phase on solute retention

The capacity factor (k') of atenolol and practolol was found to be a function of the acetonitrile and triethylamine content of the mobile phase. Changing pH of the mobile phase also substantially altered the retention

time of the drugs. Increasing the solvent strength of the mobile phase by increasing the acetonitrile content produced a concentration-dependent decrease in retention of both atenolol and practolol. The addition of triethylamine to the mobile phase produced a concentration-dependent decrease in the retention of the compounds and sharpened the peaks. These effects most probably result from the ability of triethylamine to cap the free acidic silanol groups on the silica surface. Increasing pH increased the relative retention of atenolol.

Sample preparation procedure

Bond-Elut is a family of disposable, solid-phase sample preparation columns that can process samples in a fraction of the time of traditional methods with good selectivity and reproducibility. The cyanopropyl sorbent was chosen after comparison with a reversed-phase (octadecyl) sorbent. The CN sorbent exhibited greater separation specificity than the hydrophobic phase for the extraction of atenolol. The saving in time for the analysis using Bond-Elut columns is the most important favorable factor for this method. Typically, ten samples can be processed in 5 min, ready for instrumental analysis. Since elution solvent volume is low, evaporation presents little or no problem and selective elution procedures yield final samples with fewer endogenous impurities than are usually obtained in liquid—liquid extraction methods.

Interfering substances

Several drugs which are often concurrently administered to cardiac patients were also examined for their possible interference with the quantification of atenolol (Table I). None of the drugs tested interfered in the assay.

TABLE I

CAPACITY FACTOR (k') OF DRUGS OFTEN ADMINISTERED CONCURRENTLY WITH ATENOLOL

Drug	k'
Atenolol	2.0
Practolol	3.3
Metoprolol	7.8
Quinidine	24.3
Procainamide	3.6
Disopyramide	8.0
Verapamil	3.5
Lidocaine	5.0
Timolol	6.5
Nadolol	4.0

Recovery

Under the extraction conditions chosen the absolute recovery of atenolol from heparinised human plasma was 105% and 107% at concentrations of 100 and 1000 ng/ml, respectively. These findings were obtained by direct comparison of the peak height ratios obtained on analysis of 50-μl portions of

standard to those obtained from spiked plasma extracts. However, the internal standard was added prior to the final analytical procedure to correct for any possible injection volume errors, partial evaporation or spillage, should these have occurred.

Accuracy, reproducibility and sensitivity

The combination of low detector noise following injections of plasma extracts, high extraction efficiencies and the high molar absorptivity (ϵ_{224}) permits the quantitation of atenolol in human plasma at low concentrations. The accuracy and precision of the present method were determined by assaying 400-μl aliquots of plasma containing 25, 50, 250, 750 and 1000 ng/ml of atenolol (Table II). The daily standard curves over a period of two months had an average coefficient of variation of 3.1% for atenolol (Table III). The limit of quantitation, defined as minimum signal-to-noise ratio of 4, is 25 ng/ml for atenolol; percentage difference from theoretical is 15% or less; and the coefficient of variation is less than 10%. The lower limit of detection however, was less than 10 ng/ml. These concentrations generally yielded a percentage difference from theoretical values greater than 15%. Therefore, 25 ng/ml is taken as the lower practical limit of quantitation.

TABLE II

PRECISION AND ACCURACY OF ATENOLOL MEASUREMENT BY HPLC

Atenolol added to plasma (ng/ml)	*n*	Mean of calculated atenolol concentrations (ng/ml)	S.D.	C.V. (%)	Percent difference from theoretical value
25	6	28.0	0.931	3.3	12.0
50	7	52.7	3.42	6.4	5.4
250	6	249	5.52	2.2	0.4
750	6	772	11.32	1.4	2.9
1000	6	1021	10.21	1.0	2.1

TABLE III

REPRODUCIBILITY OF STANDARDS OVER A PERIOD OF TWO MONTHS

n = 6 for each concentration.

Concentration (ng/ml)	Mean of peak height ratios	Standard deviation	Coefficient of variation (%)
25	0.0995	0.0027	2.7
50	0.1742	0.0100	5.8
100	0.2731	0.0203	7.4
250	0.7707	0.0170	2.2
500	1.4057	0.0176	1.2
750	2.349	0.0344	1.4
1000	3.103	0.0319	1.0
		Overall C.V. = 3.1%	

Analysis of five samples of each concentration of 25, 50, 100, 250, 500, 750 and 1000 ng/ml of atenolol gave a linear regression coefficient of 0.9986 for the line $Y = 0.0030258X + 0.014576$ where Y is the peak height ratio and X the concentration in ng/ml of plasma.

Practical application of the method

The current method was used to assess atenolol concentrations and β-blocking effect in subjects participating in an exercise conditioning program. Nine healthy adults (six men and three women), mean age 29 years (range 28—37 years) and mean weight 76 kg (range 53—94 kg) took atenolol, 100 mg once daily (morning) for two months. Symptom-limited maximal exercise tolerance tests were performed 7—11 h after drug ingestion five days after beginning therapy and before discontinuing therapy to assess drug effect. Blood samples were taken immediately prior to treadmill exercise. During the two-month treatment period the subjects participated in an aerobic exercise program (minimum 45 min, four times per week) designed to improve their physical fitness. A mean improvement of 22% increase in work performed to exhaustion was noted. Relevant data concerning drug effect and drug levels are shown in Table IV. The data indicate that neither chronic versus acute dosing (two months versus five days), nor physical conditioning appear to affect the degree of β-blockade or drug level obtained with atenolol.

TABLE IV

CONCENTRATION—TIME EFFECT DATA FOR ATENOLOL BEFORE AND AFTER TWO MONTHS DOSING DURING A PHYSICAL TRAINING PROGRAM

	Atenolol level (ng/ml)			Heart rate suppression			Time of treadmill test after dose (h)		
	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.	Range
Before	254	71	140—367	29.8	13.2	13—43	8.9	1.6	7—11
After	263	65	120—334	28.8	11.4	14—55	8.9	1.1	7—11

CONCLUSIONS

The method described here has been found to be useful for the measurement of plasma atenolol concentration during exercise studies and may prove useful in single-dose pharmacokinetic studies. Small sample volume combined with short chromatographic time and minimal source of interference has been achieved in the method described here which thus has considerable advantages over previously published methods.

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