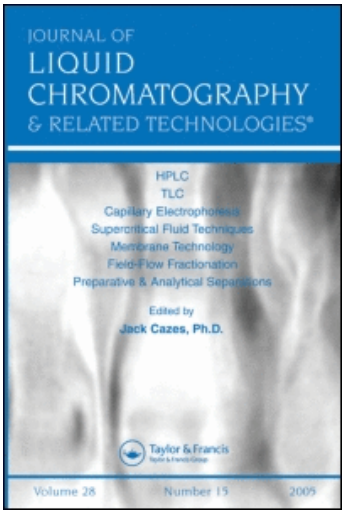

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A Liquid Chromatographic Method for the Determination of Atenolol in Human Plasma

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A LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ATENOLOL IN HUMAN PLASMA

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ABSTRACT

A reliable, highly reproducible, accurate and time-efficient high performance liquid chromatographic (HPLC) method to measure atenolol concentration in human plasma was developed and validated. Sample clean-up consists of simple and efficient liquid-liquid extraction (mean recovery 103%) which allows a high sample throughput. Chromatography on a CN-propyl column yields symmetrical and well resolved peaks for atenolol and for the internal standard (metoprolol) without any interference from endogenous plasma components. Using 1 ml plasma samples the method has a limit of detection of 12.6 ng/ml (calculated at a 99.9% confidence level) with %CV (precision) ≤ 8.8% and bias (accuracy) ≤ 3.8% for concentrations in the range of 10 - 1000 ng/ml. We now routinely use this method in human pharmacokinetic studies of atenolol dosage forms.

INTRODUCTION

Atenolol, 4-[2-hydroxy-3-[(1-methylethyl) amino] propoxy] benzeneacetamide, is a hydrophylic, relatively cardioselective ß-adrenergic receptor antagonist. Its widespread clinical use in

the treatment of hypertension and in the prophylactic management of angina required detailed pharmacokinetic characterization which in turn led to the development of numerous methods for its determination in plasma and in urine. Early gas-liquid chromatographic methods (1-12) were sensitive and specific but involved a laborious derivatization. They were largely replaced by simpler HPLC (13-26) methods using fluorimetric or UV absorbance detection. Some of these exhibit only poor chromatographic resolution with interferences coeluting with atenolol or with the internal standard (IS). Other avoid the use of an IS and are therefore prone to inaccuracies. Some reports fail to present validation data to support their statements regarding accuracy, precision and limit of detection (LD).
The procedure reported here yields base line separation of atenolol and the IS from each other and from any interferences observed in blank plasma. Validation data is presented to support the performance parameters cited for the method.

MATERIALS AND METHODS

Chemicals and Reagents

HPLC grade acetonitrile and methanol as well as Analytical grade cyclohexane, NaOH, NaH_2PO_4, Na_2HPO_4, n-butanol and H_2SO_4 were purchased from E. Merck (Darmstadt, Federal Republic of Germany). Pooled normal human plasma was purchased from the Israeli blood services (Tel Hashomer, Israel). Atenolol and metoprolol were purchased from Sigma Chemical Co. (St. Louis, Mo., USA) . HPLC grade water obtained from a NANOpure^R system (Barnstead, Dubuqe IO, USA) was used for the preparation of all aqueous solutions.

Chromatographic System

The HPLC system consisted of a SP 8800 ternary pump, a SP 4270 recording integrator and a SP 8780 autosampler (all from Spectra Physics, San Jose, CA, USA). Column temperature was maintained at 30°C by an Eldex CH-150 column heater. The effluent fluorescence was monitored with an Applied Biosystems (Ramsey, NJ, USA) 980 Spectroflow detector with excitation at 212 nm and using a 320 nm cutoff filter for the emission. The chromatography was carried out on a 125x4 mm LiChroCART^{R} cartridge preceded by a 4x4 mm precolumn, each packed with 5µm LiChrospher CN (E. Merck, Darmstadt, Federal Republic of Germany). Chromatographic data was captured to an IBM compatible personal computer using the WINner^{R} software package (Spectra Physics, San Jose, CA, USA). The mobile phase, consisting of 52% acetonitrile, 35% H_2O and 13% 0.02 M sodium phosphate buffer at pH 7.0, was pumped at a flow rate of 1 ml/min. The buffer was prepared from 0.2 M stock solutions of NaH_2PO_4 and Na_2HPO_4 and was filtered through a 0.2 µm cellulose nitrate disposable filter unit (Nalge, Rochester,NY, USA).

Preparation of standards in human plasma

Pooled, blank human plasma was spiked with a methanolic solution (172.5 µg/ml) of atenolol to give a stock solution of a final concentration of 1003.8 ng/ml. Plasma samples containing atenolol at concentrations of 500.4, 250.2, 100.5, 50.3, 20.1 and 10.0 ng/ml were prepared by serial dilutions of this stock with blank human plasma. Due to previously reported instability of atenolol in human plasma (15), only freshly prepared standards were used.

Extraction

Atenolol was extracted from thawed plasma using a modification of the procedure described by Yee (15). A 1 ml

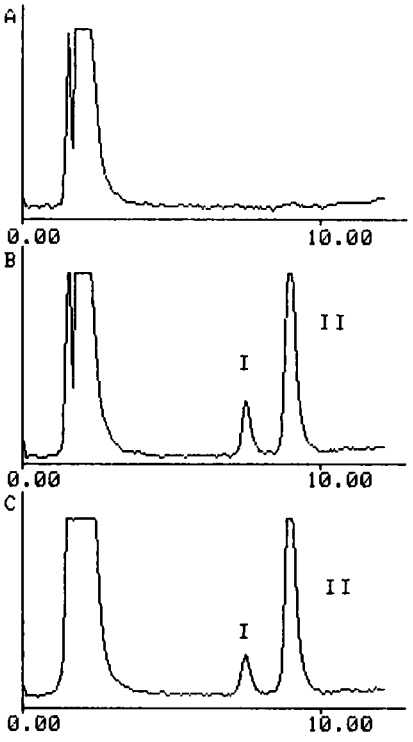

FIGURE 1: Chromatograms of: (A) blank human plasma, (B) blank plasma spiked with atenolol to a concentration of 50.3 ng/ml, (C) plasma collected 24 hours after an oral dose of 100 mg atenolol (measured level - 37.7 ng/ml). (I) atenolol, (II) internal standard.

plasma sample was pipetted into a 16x100 mm glass test tube followed by 50 µl of an IS solution (6.05 µg/ml of aqueous metoprolol), 0.25 ml of 2M NaOH and by 5 ml of a mixture composed of 45% n-butanol and 55% cyclohexane. The mixture was gently vortexed for 10 minutes. After centrifugation (10 minutes, 0°C and 3000g) the organic layer was transferred to a clean glass test tube and 250 µl of 0.05 M H_2SO_4 were added. The mixture was vigorously vortexed for 1 minute and centrifuged as above.

TABLE 1: SUMMARY OF CALIBRATION CURVE DATA *

CURVE #	SLOPE (A)	INTERCEPT (B)	r^2
1	0.01116	0.00804	0.997
2	0.00825	0.02524	0.998
3	0.00996	-0.01060	0.998
4	0.00944	0.02592	1.000
5	0.00823	-0.01324	0.998
6	0.00776	0.00032	0.997
7	0.00856	0.03488	0.998
Mean	0.00905	0.01008	-----
SD	0.00120	0.01900	-----

* Regression equation:

$$\text{Ratio of peak areas} = AxC_{atenolol} + B$$

The aqueous layer was transferred to an autosampler vial and samples of 50 µl were injected onto the HPLC column.

RESULTS

Separation

Figure 1 shows chromatograms typical for a blank plasma sample, blank plasma spiked with atenolol and a plasma sample collected from a healthy volunteer 24 hours after an oral administration of a 100 mg atenolol tablet. Atenolol and the IS eluted as symmetrical peaks (baseline level tailing factor<1.1) at 7.5 and 9 minutes respectively. No interferences with either of the above peaks were detected in blank plasma and they were resolved from each other at a baseline level (resolution factor>2).

TABLE 2: PRECISION AND ACCURACY OF ATENOLOL DETERMINATION IN PLASMA

NOMINAL CONC. (ng/ml)	OBSERVED CONC. MEAN (ng/ml)	SD	#PRECISION (%)	*ACCURACY (%)
10.0 (n=7)	9.7	0.85	8.8	-3.0
20.1 (n=7)	20.4	1.70	8.3	1.5
50.3 (n=17)	50.6	4.45	8.8	0.6
100.5 (n=7)	104.3	4.97	4.8	3.8
250.2 (n=18)	254.3	14.55	5.7	1.6
500.4 (n=6)	504.5	29.07	5.8	0.8
1003.8 (n=18)	965.9	53.72	5.6	-3.8

PRECISION = 100 x SD(OBSERVED CONC.)/MEAN OBSERVED CONC.

$$* \text{ ACCURACY} = 100 \times \frac{(\text{MEAN OBSERVED CONC.} - \text{NOMINAL CONC.})}{\text{NOMINAL CONC.}}$$

Calibration

Using the standards prepared by spiking of blank human plasma, linear calibration curves were constructed by weighted linear regression of the peak area ratio of atenolol to the internal standard versus atenolol concentration. The results obtained with 7 separate sets of standards are summarized in Table 1.

A limit of detection (LD) of 12.6 ng/ml was calculated from the above results at a 99.9% confidence level according to the procedure outlined by Massart (27), i.e.

$$LD = 6x(\text{SD of B})/(\text{Mean of A})$$

Precision and Accuracy

The precision and the accuracy of the method were determined by analyzing multiple sets of spiked plasma samples. The results presented in Table 2 indicate that the overall CV of the method ranges between 4.8 to 8.8% and the accuracy is in the range of 0.6 to 3.8%.

Recovery

The extraction recoveries were determined by measuring atenolol concentration in extracts of spiked plasma samples using atenolol standards in 0.05 M H_2SO_4 for calibration. A mean recovery of 103% (n=12, SD=7%) was observed for atenolol concentrations in the range of 10 - 985 ng/ml.

DISCUSSION

The analytical method reported here combines many of the features of methods previously published while largely avoiding most of their shortcomings and inconveniences. Some of those methods were found in this laboratory to be time consuming, others were characterized by poor chromatographic resolution and persistently contained endogenous plasma components that interfered with the peaks of interest. The extraction procedure that we have adopted is simple (up to 100 samples along with two sets of standards and controls can easily be prepared daily). The chromatographic method yields sharp, well resolved and symmetrical peaks without interferences from endogenous plasma components. Combined with high extraction recoveries this allows a low and reliably defined limit of detection without requiring large sample volumes, laborious extraction procedures or complex mobile phases containing several modifiers.

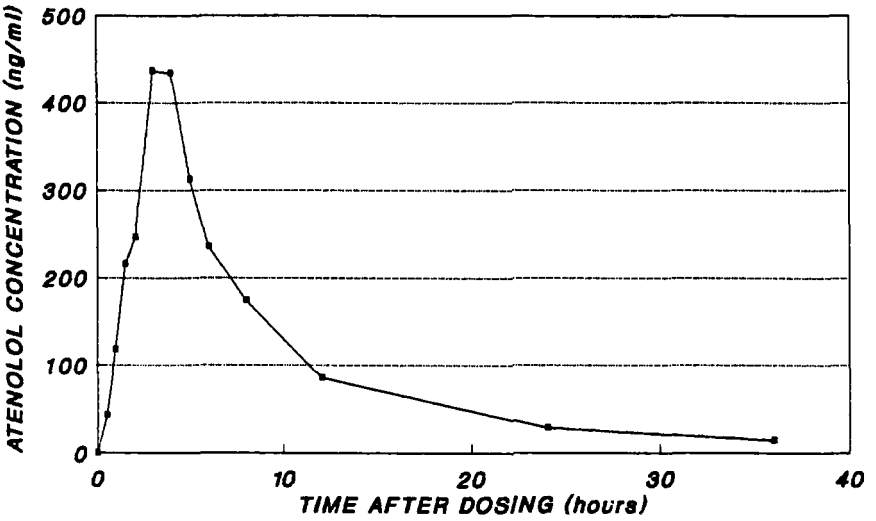

FIGURE 2: Plasma concentration of atenolol after a single oral dose of a 100 mg atenolol tablet administered to a fasting, healthy volunteer.

Some of the previously published methods state lower LD values than the one calculated here but they either fail to state the criterion used for their calculation (28) or use one that is less stringent than ours (15, 23, 24, 25). The later may be misleading in cases of poor chromatographic resolution with interfering peaks eluting in close proximity to the analyte. The detailed validation data we present demonstrates good precision and accuracy and allows independent estimation of the LD according to a clearly defined criterion. In our opinion the method described here is a practical alternative to those previously reported and it is suitable to monitor atenolol levels in pharmacokinetic, pharmacodynamic and dose optimization studies. A typical pharmacokinetic curve observed using this method is presented in Figure 2.

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