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A Sensitive High Performance Liquid Chromatography Method for Determination of Bioequivalence of Atenolol Formulations

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A sensitive and specific HPLC method with fluorescence detector has been developed for the analysis of atenolol in plasma using a reverse phase column (ODS, 250 x 4.6 mm, 10 μm). Atenolol levels were measured using its excitation and emission maxima at 276 nm and 302 nm, respectively. The method has a linearity range of 50-1000 ng/ml and recovery was found to be 95.7%. This method has been successfully used for the bioequivalence study of atenolol tablets (25 mg). C_{max}, t_{max} and AUC_{0-} of the test formulation statistically does not show significant difference from the standard formulation.

Atenolol, [4-(2-hydroxy-3-aminopropoxy) phenyl-acetamide], is a cardioselective β-adrenoreceptor blocking agent, which is used to lower blood pressure in hypertensive patients and also used in patients with myocardial infraction[1]. Pharmacokinetic parameters are largely formulation dependent. The USP states that it is compulsory to carry out bioequivalence of generic formulations, before introducing to the market[2]. It is also mandatory to confirm the release pattern if any excipient is changed in the existing formulation both by dissolution and bioequivalence studies. Atenolol is reported to have a variation of four folds in the maximum blood concentration[3]. Reports on elimination half life also show values ranging from 3.8 h[4] to 7.0 h [5]. Its effective plasma concentration ranges from 0.1 to 1 μg/ml[6].

Several analytical methods for quantifying atenolol in biological fluids have been reported, which include spectrofluorimetry[7], high performance liquid chromatography[8], dual column liquid chromatography with fluorimetric detection[9], gas liquid chromatography with electron capture detection[10,11] and thin layer chromatography with fluorimetric detection[12]. In the spectrofluorimetric method, atenolol is extracted from alkaline solution with ethyl acetate and fluorescence is determined after reextraction in a solution containing sodium dihydrogen orthophosphate in a cuvette. The disadvantage of this technically simple method is relatively high detection limit of 50 ng/ml.

The detection limits of the gas chromatographic methods are about 10-20 ng/ml. It has been possible only with these methods to measure plasma levels of atenolol for sufficiently long periods after administration of therapeutic doses (50-100 mg). However, both methods require derivatization of atenolol with heptafluorobutyric anhydride. High performance thin layer chromatographic method using fluorescence detection mode showed detection limit as low as 5 ng/ml for atenolol, but recovery of the drug is only 64.6±5%. Therefore, it is necessary to develop simple, specific, sensitive and accurate method for estimation of atenolol in plasma.

The proposed method consists of a selective two step extraction of atenolol followed by HPLC with fluorescence detection. The method is rapid, specific, sensitive and accurate. This method was successfully used for a bioequivalence study.

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EXPERIMENTAL

Atenolol was obtained from Sigma Chemicals, USA. Test Formulation (T) and the reference formulation (R) of atenolol (25 mg tablets) were obtained from the market. n-Hexane and n-butanol (Analytical Grade, E. Merck, India) were used for extraction. HCl (35% w/v, Analytical Grade, E. Merck, India), sodium chloride and sodium hydroxide (EP, S.D. Fine Chemicals), methanol (HPLC Grade, Glaxo, India) and double distilled water were used for preparation of the mobile phase.

Chromatographic Parameters:

HPLC was performed in a system consisting of Rheodyne injection system, isocratic pump (LC-250 Perkin Elmer), bonded phase column (Perkin Elmer ODS 250 mm x 4.6 mm, 10 µm) and fluorescence detector (Hitachi F-2000). The excitation and emission maxima for atenolol were 276 nm and 302 nm, respectively.

The mobile phase was prepared by mixing 4.25 ml of HCl and 5.85 g of sodium chloride in 1000 ml of distilled water (0.1 M NaCl solution). This solution was mixed with methanol in the proportion of (7.5:2.5 v/v). The flow rate of the mobile phase was 1 ml/min. Aliquot (20 µl) of the extracted solution was injected directly into the column. A stock solution of atenolol (1 mg/ml) was prepared in water. Appropriate dilutions were made by diluting the stock solution with water.

Analytical procedure:

One millilitre of plasma samples (drug free plasma, plasma containing a known amount of drug and unknown plasma samples) were placed in 15 ml screw capped centrifuge tube. To each tube, 0.2 ml of 2 N sodium hydroxide and 5 ml of n-hexane: n-butanol mixture (55:45 v/v) were added. The tubes were vortexed for 1 min and centrifuged for 10 min at 2000 rpm. Separated organic phase was transferred to a second centrifuge tube containing 1 ml of 0.05 M hydrochloric acid and vortexed for 1 min. It was centrifuged for 5 min at 2000 rpm. Twenty microlitres of lower aqueous phase was injected into the chromatographic system.

Calibration curves were obtained by plotting the peak height of atenolol against the concentration over a range of 50-1000 ng/ml. The concentration of unknown sample was determined using calibration curve.

Method Validation:

The recovery of atenolol from plasma was determined by comparing peak height obtained from spiked plasma with atenolol at concentration of 50, 100, 200, 500 and 1000 ng/ml with the peak height obtained with the standard atenolol solution. The intra-day precision was determined by analyzing plasma samples spiked with atenolol at 100, 200 and 500 ng/ml on the same day. The inter-day precision was determined by analyzing 100, 200 and 500 ng/ml spliked plasma samples daily for 5 days. Linearity of the detector response was tested by analyzing standards 5 times for each concentration ranging between 50-1000 ng/ml.

Pharmacokinetic studies:

Six healthy male volunteers, aged 18 to 35 years, weighing 43-54 kg, participated in the study. The written, informed consent was taken from all volunteers and protocol of the study was approved by the local ethical committee. One test formulation (T) and one reference formulation (R) of atenolol (25 mg) were taken for bioequivalence studies. The volunteers were overnight fasted and continued fasting until 4 h post dose, but allowed free access to water. Each volunteer received single oral dose of 2 x 25 mg tablets in a single blind cross over fashion with one week wash out period in a predetermined random order. No other drugs were taken by volunteers two weeks before and during the study.

Blood samples were collected from antecubital vein into heparinised tubes at 0, 0.5, 1.0, 2.0 3.0, 4.0, 6.0 and 8.0 h after the drug was given and were immediately centrifuged at 3000 rpm for 15 min. Separated plasma samples were stored at -20° until analyzed.

The peak plasma concentration (C_{max}) and time to peak concentration (t_{max}) were obtained from the plasma concentration - time data. The $AUC_{0-\infty}$ values were calculated by linear trapezoidal method, K_{el}, $t_{1/2e}$, $t_{1/2a}$ were calculated by using a computer program written in FORTRAN - 77. The program was validated by manual calculation of one set of data. The pharmacokinetic parameters ($AUC_{0-\infty}$, t_{max}, C_{max}) for test formulation were compared with those of reference formulation using a student's paired t-test and ANOVA.

RESULTS AND DISCUSSION

Several analytical methods have been reported for estimation of atenolol. However, most of the methods are not suitable for determination of atenolol in biological fluids. This is due to lack of enough sensitivity for detection of low levels of atenolol in biological fluids and

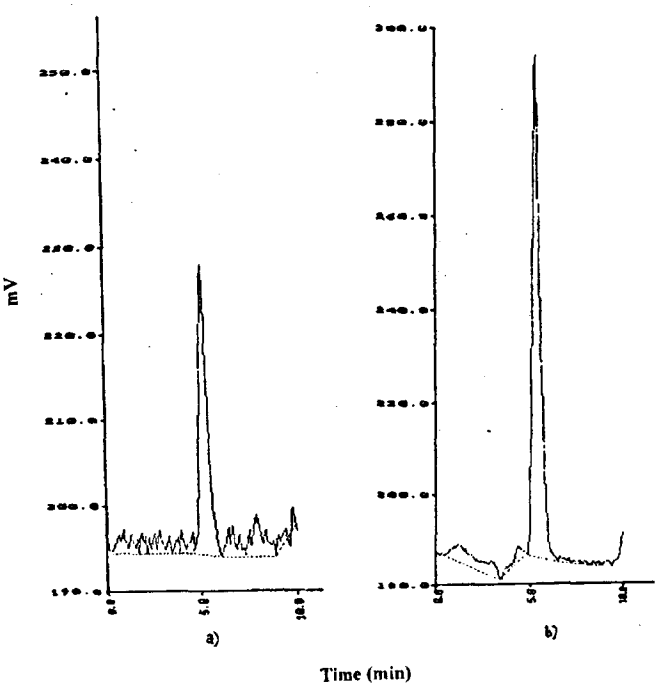

Fig. 1: Chromatogram for atenolol in mobile phase and volunteer plasma sample

Chromatogram showing response for a) atenolol (200 ng/ml) in the mobile phase; b) atenolol in the plasma sample collected 2 h after administration of atenolol tablets (Formulation R) to human volunteer, by proposed HPLC method

specificity. Gas chromatographic methods have the required sensitivity but they need derivatization of atenolol prior to its estimation. HPLC method was selected owing to its simplicity, specificity, sensitivity and reproducibility. It also provides rapid analysis with minimum amount of sample.

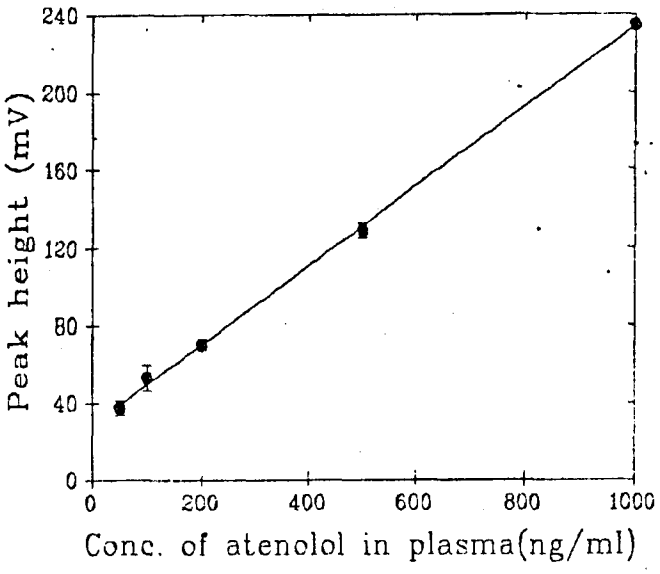

Fig. 2: Calibration curve for atenolol spiked in plasma

Calibration curve for atenolol spiked in plasma plotted as peak height (mV) v/s concentration of atenolol (ng/ml) by proposed HPLC method

Extraction procedure always remains quite crucial to obtain good recovery of analyte from plasma sample. In the proposed procedure plasma sample was alkalinized and extracted with n-hexane:n-butanol (55:45 v/v) mixture. Atenolol from the organic extract was further transferred to acidic medium by extracting it with 0.05 M HCl. Thus, a simple two step extraction procedure was developed for effective extraction (average recovery of 95.67%) of the drug from plasma.

By using octadecylsilyl (ODS i.e. C18) column and a mixture of methanol and NaCl solution as the mobile

TABLE 1 : ACCURACY OF ATENOLOL MEASUREMENT BY PROPOSED HPLC METHOD

Atenolol spiked in plasma (ng/ml)	Concentration found*(ng/ml) (Mean±S.D.)*	% Accuracy[a]
50	49.6 ± 2.80	98.28
100	98.8 ± 2.24	98.80
200	197.7 ± 1.86	99.85
500	498.1 ± 2.53	99.62

*Average of 5 determinations, [a]after correction for recovery

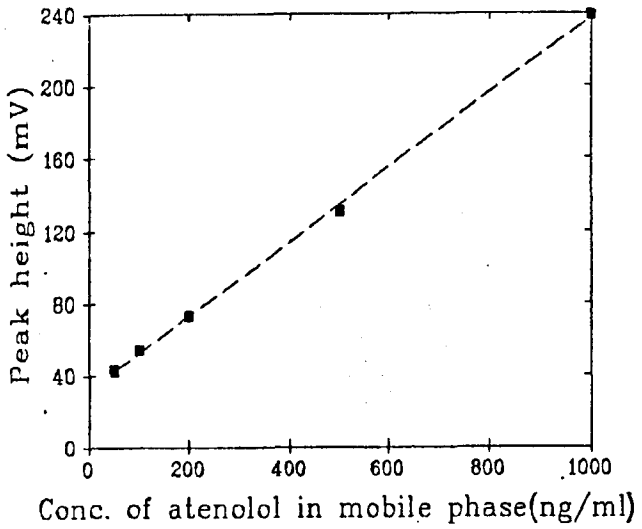

Fig. 3: Calibration curve for atenolol in mobile phase

Calibration curve for atenolol in mobile phase plotted as peak height (mV) v/s concentration of atenolol (ng/ml) by proposed HPLC method

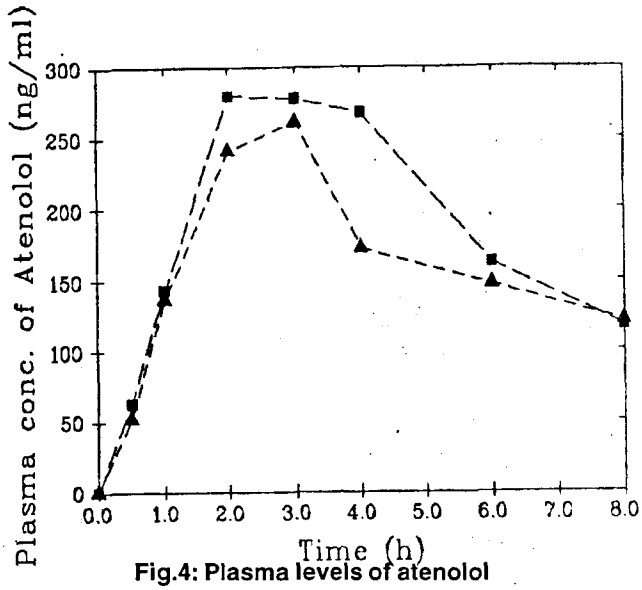

Fig.4: Plasma levels of atenolol

Average plasma concentration (ng/ml) v/s time (h) profiles after administration of atenolol formulations (2 x 25 mg tablet) to six healthy human volunteers (■—■, Formulation T; ▲—▲ Formulation R)

TABLE 2 : RECOVERY OF ATENOLOL FROM SPIKED PLASMA

Atenolol spiked in plasma (ng/ml)	% Extracted*
50	88.06
100	97.74
200	96.03
500	98.39
1000	98.13
Average % recovery for Atenolol	95.67

*Average of 5 determinations.

phase it was observed that atenolol was separated well from the endogenous plasma components at the flow rate of 1 ml/min. The fluorimetric detector enhanced the peak response and specificity to detect atenolol in lower concentration range. The detection of atenolol was carried out at excitation and emission wavelengths 276 and 302 nm, respectively. The retention time for atenolol was found to be about 5.3 min (Fig. 1).

The least square linear regression analysis of the peak height (y) versus concentration of atenolol in plasma (x), obtained by assaying plasma samples spiked with atenolol over the range of 50 to 1000 ng/ml, gave equation of the straight line y=0.2044 x + 28.9709, with a correlation coefficient of 0.9996 (Fig. 2). Similarly, solutions

TABLE 3 : PRECISION OF METHOD FOR DETERMINATION OF ATENOLOL

Sr. No.	Conc. of atenolol in plasma (ng/ml)	Mean conc. of atenolol found (ng/ml)*		% C.V.	
		Intra day	Inter day	Intra day	Inter day
1	100	100.4	101.9	1.01	1.71
2	200	194.2	204.5	0.86	1.09
3	500	494.3	491.8	0.64	1.39

*Each value represents a mean of five determinations.

TABLE 4 : PHARMACOKINETIC DATA FOR ATENOLOL FORMULATIONS

Pharmacokinetic parameter	Formulation R Mean±S.D.*	Formulation T Mean±S.D.*	Reported value	Reference
Elimination rate constant K_{el} (h^{-1})	0.0958±0.018	0.2695±0.0239	0.147	13
Elimination half life $t_{1/2e}$ (h)	7.51±1.38	2.621±0.34	3.8	4
			4-14	5
			4.955	13
Absorption rate constant K_a/h^{-1}	1.2346±0.53	0.5738±0.103	-	-
Absorption half life $t_{1/2a}$ (h)	0.6873±0.28	1.2506±0.218	0.8	4
Time to peak plasma conc. t_{max} (h)	2.5277±0.77	2.55±0.39	2.5	4
Maximum plasma conc. C_{max} (ng/ml)	174.92±53.1	215.97±37.39	358	13
Clearance Cl (L/h)	0.02407±0.0095	0.03223±0.0036	2.4±0.2 ml/min/kg	14
Volume of distribution Vd (L)	0.2428±0.066	0.1291±0.022	0.5-1.5L/kg	14
Mean Residence Time MRT (h)	131.52±53.45	131.57±24.99	-	-
AUC_{0-8} (h.ng/ml)	1296.45±491.0	1523.57±251.3	1916	13
$AUC_{0-\infty}$ (h.ng/ml)	2697.25±1350.6	1915.66±356.23	3210	13

*Each value is the mean±standard deviation of six observations

containing known quantity of atenolol prepared in the mobile phase were assayed. The least square linear regression evaluation of the peak height (y) versus concentration of atenolol (x) gave the equation of the line, y=0.2055 x + 31.6956 and the correlation coefficient was 0.9998. The linearity range in both the cases was found to be 50 to 1000 ng/ml for atenolol (Fig. 3). The limit of quantitation and limit of detection were found to be 50 ng/ml and 10 ng/ml, respectively.

Accuracy of the measurement of atenolol in plasma was 98.28 to 99.85% (Table 1). Overall average recovery of atenolol from plasma was found to be 95.67% (n=5) (Table 2). Selectivity of the method was ascertained by the fact that no endogenous plasma components interfere with atenolol peak (Fig. 1). The method was validated by determining reproducibility and accuracy for spiked plasma samples. Intra-day and inter-day coefficients of variation for analysis of the plasma samples on the same day and on five days over a period of one week varied from 0.639 to 1.01% and 1.09 to 1.71%, respectively (Table 3).

Proposed HPLC assay method for atenolol provides reproducible estimates of the drug concentration in volunteer plasma samples with sufficient sensitivities to allow pharmacokinetic and bioequivalence study. There is no significant difference in the average t_{max} values of atenolol for reference formulation (2.527±0.77 h) and test formulation (2.550±0.39 h) (Table 4, Fig. 4). Similarly, average C_{max} values for both the formulations are not significantly different.

Based on log transformed data of C_{max} and $AUC_{0-\infty}$ for formulation R and T, at the 95% confidence interval the

ratio (T/R) was found to be 0.97 to 1.06 for C_{max} and 0.92 to 1.00 for $AUC_{0-\infty}$. It is well within the limits of USP guidelines (0.80 to 1.25)[2].

Statistical evaluation of the pharmacokinetic parameters such as C_{max}, t_{max} and $AUC_{0-\infty}$ of atenolol for the two formulations after oral administration provided sufficient evidence for bioequivalence between these formulations.

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