# Determination of Atenolol in Human Plasma by New HPLC Method with Fluorescence Detection for Pharmacokinetics Studies

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## ABSTRACT

**Purpose:** A new, sensitive, specific and precise HPLC analysis method was developed for the determination of atenolol in human plasma in order to be utilized for pharmacokinetics study. **Methods:** The drug was extracted from plasma by liquid-liquid extraction technique using dichloromethane: 2-propanol (75:25). Bamethan sulfate was used as internal standard (IS). Samples were analyzed on ODS-3 $C_{18}$ Intertsil column (150 x 4.6 mm, 5 µm), applying triethylamine (0.5%): methanol at a ratio of 90:10v/v with a final pH of 3.5 in isocratic mode as a mobile phase at a flow rate of 1.3 ml/min to attain adequate resolution. Using a Spectra autosampler, separations were performed at room temperature and monitored at an excitation wavelength of 228 nm and an emission wavelength of 298 nm after injection a 60µl sample into the HPLC system.

**Results:** A peak area was obtained for atenolol and bamethan with 6.4 and 10.4min retention time, respectively. The lower limit of quantitation (LLOQ) was identifiable and reproducible at 10ng/ml with a precision of 1.754%. The intra-day and inter-day precision at 30, 400 and 700 ng /ml level was found to be 1.909%, 1.571% , 1.358% and 3.229, 1.471, 3.246 respectively, always lower than the accepted criteria limits (15%). The relative recovery% of atenolol at 30, 400, and 700ng/ml was found to be 100.733, 99.948, and 98.599 respectively. **Conclusions:** The analysis method was found to be sensitive, accurate, and precise for the quantification of atenolol in human plasma. It was applied successfully, for pharmacokinetics studies.

**INTRODUCTION**

Atenolol is a synthetic, beta$_1$-selective (cardio selective) adrenorecetor blocking agent with low lipid solubility. Chemically, it is described as benzeneacetamide, 4 -[2'-hydroxy-3'-[(1- methyl ethyl) amino] propoxy]. Its empirical formula is: $C_{14}H_{22}N_2O_3$; M.wt = 266.34 and its structural formula is (1, 2).
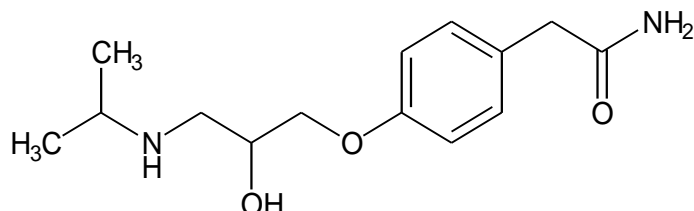


Atenolol is indicated in the management of hypertension, angina pectoris, cardiac arrhythmias and myocardial infarction It may be used alone or concomitantly with other drugs (1,3).

In man, absorption of an oral dose is rapid and consistent but incomplete. Approximately 50% of an oral dose is absorbed from the gastrointestinal tract, the remainder being excreted unchanged in the feces. Peak plasma levels are reached in 2 to 4 hours after ingestion. Atenolol plasma concentrations increase proportionally to the dose. In bioavailability (BA) studies in healthy subjects in which the area under the curve  (AUC) after orally and intravenously administration of atenolol was compared, an absolute BA of 40–60% . Atenolol undergoes little or no metabolism by the liver, and the absorbed portion is eliminated primarily by renal excretion. Only a small amount (6%-16%) is bound to proteins in the plasma. The elimination half-life of oral atenolol is approximately 6 to 7 hours, and there is no alteration of the kinetic profile of the drug by chronic administration (3,4).

To date several methods have been developed for the quantification of atenolol either alone or in combination with other drugs in different matrices. Atenolol has been determined by liquid chromatography-mass spectrometry (LC/MS) in human plasma after its extraction by solid phase technique (5). HPLC methods have also been reported for the determination of atenolol alone in human plasma (6, 7) and in pharmaceutical preparations (8). The simultaneous quantification of atenolol with indapamide in pharmaceutical dosage forms, human blood and milk (9), with chlorthalidone in spiked human plasma (10), with amlodipine besylate and aspirin in

bulk drug and formulation (11), with atorvastatin ,aspirin and losartan in pharmaceutical drug products (12) were reported.

In spite of the presence of many analytical methods for atenolol but, still there is an increasing interest for development of more accurate, simple and rapid method for analysis of atenolol especially in human plasma.

The aim of this study is to develop and validate a new, sensitive, accurate and reproducible reversed-phase HPLC with fluorescence detection method for determination of atenolol in human plasma in order that it could be applied for the determination of pharmacokinetic parameters following oral dosing.

## MATERIALS AND METHODS

### Materials and reagents

Tenormin® 100mg Atenolol tablets (Astra Zeneca, United Kingdom), Atenolol standard powder, Bamethan sulfate standard powder, Dichloromethane (Scharlau, Spain), Isopropanol (Scharlau, Spain), Methanol HPLC grade (Acros, Belgium), Milli-Q HPLC water (Millipore, France), Phosphoric acid (Panreac, Spain), Potassium carbonate, (Scharlau, Spain), Triethylamine (Scharlau, Spain).

### Instruments

Waters isocratic pump 515 (USA), Spectra system FL 3000 fluorescence detector (USA), Spectra system AS 3000 autosampler (USA),Chromoquest 3.0 ® software (USA), GFL 3006 shaker (England), Stuart scientific vortex shaker (England).

### Preparation of standard solutions

Stock solutions of atenolol and its internal standard bamethan sulfate, each 100µg/ml were prepared in water (using atenolol and bamethan sulfate standard powder). Working standard solutions of atenolol were prepared by serial dilution of stock solution using water to attain a concentration of 0.5, 1.0, 5.0, 15.0, 25.0 and 45.0µg/ml, keeping internal standard at a concentration of 20µg/ml in each one. All solutions were prepared daily.

### Plasma sample preparation for calibration curve

The human blood samples were transferred to heparinized tubes and then immediately centrifuged at 4000 rpm for 10 min.  The plasma was separated by

polypropylene disposable tips and transferred to eppendorf tubes and then immediately stored at −20°C ± 2 in the deep freezer until analysis.

The calibration curve of atenolol in plasma was constructed by spiking 980µl of plasma samples (which was first thawed at room temperature) with 20µl of the previously prepared working standard solutions. Accordingly, the plasma samples contain a final concentration of atenolol equivalent to 10, 20, 100, 300, 500 and 900ng/ml respectively and 400 ng/ml of internal standard. While blank plasma samples were spiked with 20µl of water.

The samples were vortex- mixed for 30 sec. to be applied to the liquid–liquid extraction procedure.

**Quality Control (QC) samples preparation**

Working standard solutions of atenolol were prepared by serial dilution of stock solution (100µg/ml) using water to attain a concentration of 1.5, 20.0 and 35.0µg/ml for atenolol Quality Control (QC) samples preparation, keeping internal standard at a concentration of 20µg/ml in each one. All solutions were prepared daily. Quality Control samples were prepared by spiking 980µl of plasma samples (which was thawed at room temperature) with 20µl of the freshly prepared working standard solutions (1.5, 20.0, and 35.0µg/ml of atenolol, with 20µg/ml of bamethan sulfate). Accordingly, the plasma samples contain a final concentration of atenolol equivalent to 30, 400, and 700ng/ml respectively and 400 ng/ml of bamethan sulfate as IS.

**Sample preparation by liquid-liquid extraction**

Drug was extracted from plasma samples using liquid-liquid extraction technique. Potassium carbonate buffer (150 µl of 1M, pH=11) was added to one ml of each spiked plasma sample for protein precipitation. The samples were then vortex-mixed for 1min. After that, samples were extracted with 5 ml dichloromethane: isopropanol (75: 25) and shaken for 20 minutes. The samples were centrifuged at 4000 rpm for 10 minutes and the organic layer was separated and evaporated at 50ºC under nitrogen stream. Finally, the residue was reconstituted with 200 µl of mobile phase and then vortex-mixed for 15 sec, then put it in insert tubes inside the pre-labeled HPLC vials and 60 µl were injected into the analytical column using the HPLC auto sampler (Figure1).
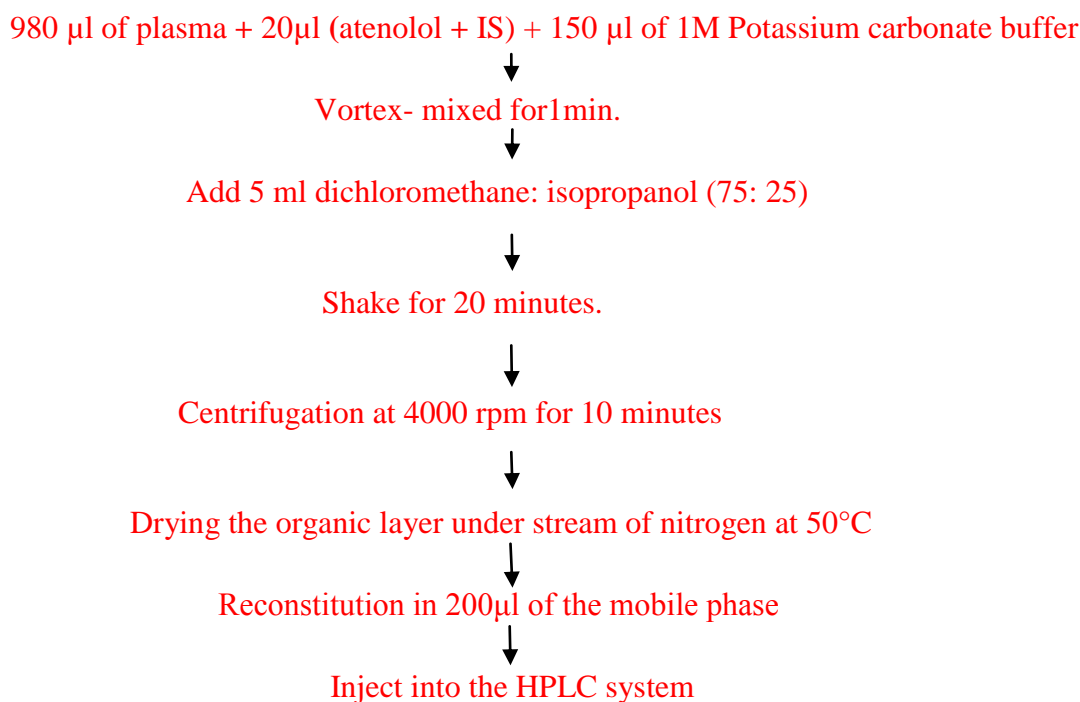
980 µl of plasma + 20µl (atenolol + IS) + 150 µl of 1M Potassium carbonate buffer

↓

Vortex- mixed for1min.

↓

Add 5 ml dichloromethane: isopropanol (75: 25)

↓

Shake for 20 minutes.

↓

Centrifugation at 4000 rpm for 10 minutes

↓

Drying the organic layer under stream of nitrogen at 50°C

↓

Reconstitution in 200µl of the mobile phase

↓

Inject into the HPLC system

**Fig. 1: Scheme for extraction procedure of atenolol from human plasma.**

**Chromatographic conditions**

A specific high performance liquid chromatographic (HPLC) assay using Fluorescence detection and Bamethan as Internal Standard was developed for the determination of atenolol in human plasma. The different HPLC experimental parameters were optimized. The optimized chromatographic conditions were :

- Column: Intertsil ODS-3 $C_{18}$ 5 µm (150 x 4.6 mm).

- Mobile phase: 0.5% triethylamine : methanol in a ratio of 90:10 (v/v)

- Detection: Fluorescence detector set at an excitation wavelength λ of 228 nm and an emission wavelength λ of 298.

- Flow rate: 1.3 ml/min.

- Injection volume: 60 µl.

- Autosampler temperature: ambient.

The mobile phase was always degassed and clarified by filtration through porous membranes with 0.45µm pore size. A mobile phase degasser was connected on line during the analysis runtime, then pumped at a flow rate of 1.3 ml/min, in isocratic mode on the column.

The sample (60µl) was injected into HPLC system and the data was acquired employing Chromquest 3.0® Workstation Software.

**Method development**

The combination of samples liquid-liquid extraction and HPLC were developed to provide a rapid assay and a valid analytical method for the determination of atenolol, free from interfering with endogenous plasma components and to obtain adequate resolution. Separations were performed at room temperature.

**Validation of the method**

The proposed analytical method was validated according to standard guidelines (13 – 16) with respect to the following parameters.

**Linearity**

The linearity of the proposed method was established from the standard calibration curve constructed at several concentration levels (10.0-900ng/ml of atenolol with constant concentration of its IS (Bamethan sulfate at 400ng/ml), for six consecutive days. Calibration curve were constructed for atenolol in the spiked plasma samples by plotting the relative peak area (ratio of peak area of drug to peak area of IS) against their respective concentrations using a linear least squares regression analysis. In addition, a blank and a zero sample were prepared to confirm the absence of interferences.

**Specificity/selectivity**

The specificity/selectivity of the analytical method was investigated by confirming the complete separation and resolution of the required peak area of atenolol from its IS (bamethan) in human plasma samples spiked with appropriate concentration of these compounds.

The method's specificity was determined by screening six different batches of healthy human plasma. The tests were accomplished to ensure absence of interfering from endogenous plasma components.

**Accuracy and Precision**

**Intra-day accuracy and precision**

The intra-day precision and accuracy of the assay was measured by analyzing five spiked samples of atenolol at three different concentrations (30, 400 and 700 ng/ml); the concentrations were calculated by applying the regression equation of the calibration curve. The deviation of the mean from the true value serves as the measure of accuracy. The precision and accuracy deviation values should be within 15% of the actual values except at LLOQ where it shouldn't deviate by more than 20%. The statistical evaluation includes means, standard deviation, coefficients of variation, accuracy, and relative errors (RE %).

**Inter-day accuracy and precision**

The inter-day precision was done at three different concentrations (30, 400 and 700 ng/ml) over three days, the concentrations were measured by analyzing forty five samples (five determinations from each concentration per day) and were calculated applying the regression equation of the calibration curve. The statistical evaluation includes means, standard deviation, coefficients of variation, accuracy, and relative errors (%).

**Accuracy and precision for Quality Control samples**

The accuracy and precision for Quality Control samples were demonstrated by analyzing over two days duplicates of Quality Control sample at three concentration levels representing the entire range of the standard calibration curve. The low QC samples (30 ng/ml) were designed to be three times the LLOQ (10 ng/ml), while the mid QC samples were taken at the center (400 ng/ml) and the high QC samples were taken near the ULOQ (700 ng/ml).

**Recovery**

The absolute peak area (detector response) obtained from the injections of the prepared plasma standards were compared to the absolute peak area (detector response) of an equivalent pure authentic standard, which was prepared to contain a drug concentration assuming 100% recovery. The absolute recoveries were calculated for both Atenolol and internal standard by comparing peak areas of the extracted samples with the unextracted pure authentic standard solutions peak areas, while the relative recovery was determined for Atenolol comparing the calculated

concentrations of extracted samples to their respective nominal values. Both absolute and relative recoveries of Atenolol were measured at three concentration levels (30, 400 and 700 ng/ml).

**Sensitivity**

The lowest standard concentration in the calibration curve is considered as the lower limit of quantitation (LLOQ), and should meet the following criteria:

- LLOQ response is five times the response of the blank.
- LLOQ response is identifiable, discrete and reproducible with precision of 20% and accuracy of 80-120%.

The peak is identifiable, precise and accurate at this concentration. The lower limit of quantitation of atenolol in plasma was considered to be 10.0ng/ml.

**Stability**

Sufficient aliquots of human plasma were spiked with atenolol to reach the final concentrations of 30, 400 and 700 ng/ml. Five determinations were assigned soon after aliquot preparation for initial concentration determination for each aliquot. Samples were extracted and then analyzed by the proposed method and their concentrations were determined.  The following allocation for each of spiked aliquots was applied:-

*Short-term stability*

Five samples from each of the stored plasma aliquots were thawed and kept at room temperature for a period of time exceeded that expected to be encountered during the routine sample preparation (around 6 hrs). Samples were extracted and then analyzed.

*Post-preparative stability*

The autosampler stability was conducted by preparing 10 determinations from each of the stored plasma aliquots after thawing and extraction. The processed samples were pooled and 5 measurements were initially done. The remaining pooled processed samples were kept under the autosampler conditions (ambient) for a period of time more than that expected during the analysis run time (24 hrs) and then analyzed.

*Freeze and thaw stability*

Testing for freeze and thaw atenolol stability was determined through three freeze and thaw cycles. Five samples from each of the stored plasma aliquot were thawed completely unassisted at room temperature and refrozen at the same conditions ($-20^oC$). This cycle was repeated two more times. Samples were then extracted and analyzed.

*Long- term stability*

Samples from each of the aforementioned plasma aliquots were stored to perform long-term stability analysis at $-20^oC$. The total storage period exceeded the time between the date of volunteers' first sample collection and date of last sample analysis. After samples analysis, the concentrations of all stability samples were compared to the mean of that calculated initially at the first day.

*Stock solutions stability*

All stock solutions were prepared daily. The stability of atenolol and internal standard stock solutions were evaluated by testing their validity for 6 hours at room temperature. Stability of stock solutions was expressed as % recovery.

*Atenolol stock solution stability*

Stability of Atenolol stock solution was evaluated using triplicate injections. 30 µl of Atenolol stock solution were diluted with mobile phase up to 1500 µl (30: 1500). 10 µl of this solution was injected into the HPLC column soon after preparation. The stock solution was kept at room temperature for 6 hours and then re-injected into the HPLC after being diluted with mobile phase up to 1500 µl (30: 1500). Mean peak areas of the late injected stock solution were compared to those injected freshly.

*Internal standard stock solution stability*

Stability of internal standard stock solution was evaluated using triplicate injections. 30 µl of stock solution were diluted with mobile phase up to 1500 µl (30: 1500). 10 µl of this solution was injected into the HPLC column soon after preparation. The stock solution was kept at room temperature for 6 hours and then re-

injected into the HPLC after being diluted with mobile phase up to 1500 µl (30: 1500). Mean peak areas of the late injected stock solution were compared to those injected freshly.

**Pharmacokinetics study design, drug administration and sample collection**

The proposed analytical method was developed and validated to be utilized for atenolol determination in plasma samples of volunteers applied for pharmacokinetics study.

Ten healthy adult male volunteers participated for this study. Their mean age was $30.0 \pm 5.52$ years with a range of $25 - 40$ years. The volunteers did not have any significant cardiac, hepatic, renal, GI, or hematological disease by their medical history, physical examination and routine laboratory tests. Subjects were instructed to abstain from taking any drug including over-the-counter (OTC) for two weeks prior to and during the study period. The drug was administered as a single oral dose of 100mg tenormin tablet with 240 ml of water after an overnight fasting of 12 hr.

Blood samples (7ml) were withdrawn via an Indwelling Cannula. The blood was sampled according to the following time schedule: At zero time (30 min. before dosing), and then at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 6.0, 8.0, 12.0, 16.0, 24.0, 30.0 and 36.0 hours post dosing (a total of 16 blood samples were collected from each volunteer).

The blood samples were transferred to heparinized tubes and then immediately centrifuged for 10 min at 4000 rpm. The plasma was separated and then stored at $-20°C \pm 2$ in the deep freezer until analysis by the proposed analytical method.

Before extraction, clinical samples were spiked with internal standard only. For this purpose, 20 µl of solution containing 20 µg/ml of internal standard were added to each plasma sample (980µl). Clinical plasma samples of each volunteer were analyzed with their own calibration curve and QC samples as one batch in a single run.

**Data analysis for pharmacokinetic (PK) calculations**

- ❖ The following PK parameters: $C_{max}$ , $t_{max}$ , $AUC_{0-t}$ , $AUC_{t-\infty}$ , $AUC_{0-\infty}$ , $C_{max}/AUC_{0-\infty}$ , $\beta$ and $t_{0.5}$ were calculated for each subject and for each period applying non-compartmental analysis (17).
- ❖ The % extrapolated AUC will be calculated as $(AUC_{t-\infty}/AUC_{0-\infty})$ X 100. The value of (% extrapolated AUC) should not exceed 20% of the total AUC value $(AUC_{0-\infty})$ (17).
- ❖ The terminal elimination rate constant ($\beta$) was

estimated for each subject and for each period via linear regression of the last points (at least three points) of the terminal phase of the log-concentration versus time curve (17).

❖           The values of $C_{max}$ and $t_{max}$ were obtained directly by visual inspection from concentration versus time curves of individual volunteers.

❖           The mean drug concentration $\pm$ standard in plasma vs. time data was plotted in the rectilinear and semilog graph types.

**Definitions of the PK parameters (17)**

$C_{max}$   = Maximum concentration of drug in plasma.

$t_{max}$   = Time to attain $C_{max}$ .

$AUC_{0-t}$ = Area under the plasma concentration-time curve from time zero to $t_{last}$, calculated by Trapezoidal rule.

$AUC_{t-\infty}$ = Extrapolated Area ($AUC_{Extrapolated}$) which is the Area under the plasma concentration-time curve from $t_{last}$ to infinity, calculated as $C_{last}/\beta$. It is also called residual Area ($AUC_{Residual}$)

$AUC_{0-\infty}$ = Total Area under the plasma concentration-time curve from time zero to infinity, calculated from the sum of $AUC_{0-t} + AUC_{t-\infty}$.

$\beta$   = First order terminal elimination rate constant.

$t_{0.5}$ = First order terminal elimination half-life which is equal to $0.693/\beta$

**RESULTS AND DISCUSSION**

**Linearity**

The linearity of the method was evaluated from the calibration curve of spiked plasma samples at several concentration levels of atenolol (constructed for six consecutive days). The mean relative peak area (ratio of peak area of the drug to the peak area of its IS) was yielded a linear correlation over a concentration range of 10 – 900ng/ml. A typical calibration curve of spiked plasma samples with the regression equation and their respective correlation coefficient ($R^2$) of atenolol is shown in figure 2.
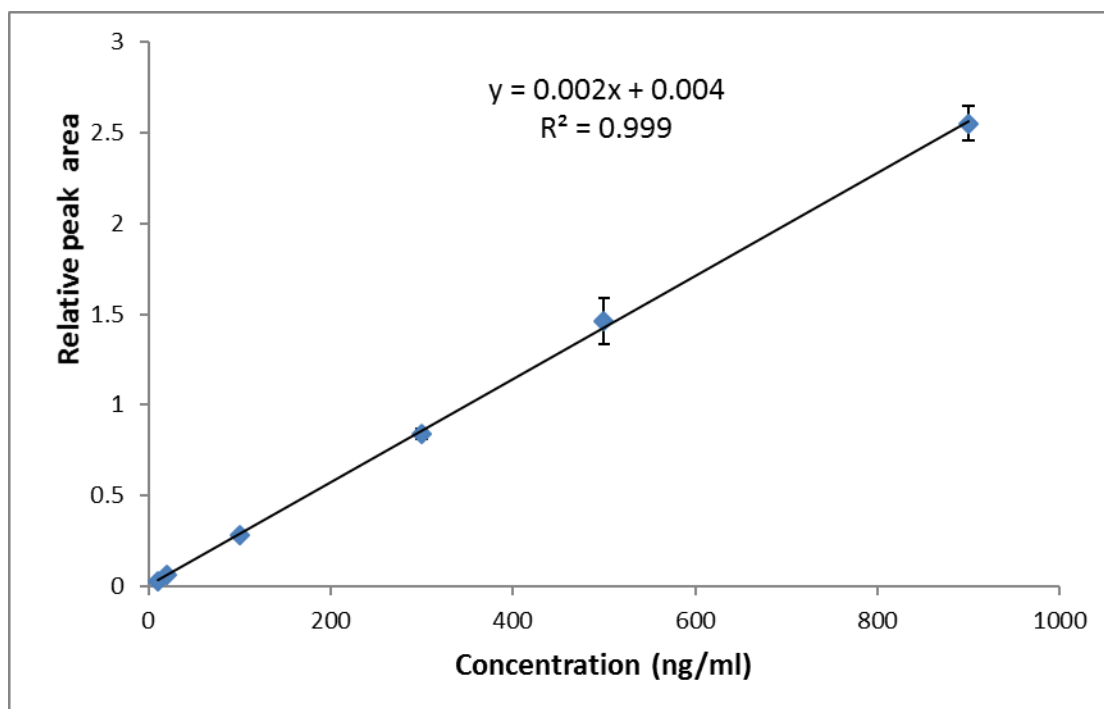
**Figure 2: Calibration curve of atenolol with bamethan sulfate as internal standard in human plasma.**

### Specificity/selectivity

Representative chromatogram of blank plasma was confirmed the presence of very little interference from the endogenous component (Figure 3). Chromatograms of spiked plasma samples of atenolol at concentration ranging from 10–900ng/ml with its IS at a constant concentration (400ng/ml) confirming that atenolol and its IS bamethan sulfate were well resolved and completely separated at retention times of 6.4 and 10.4min respectively, as shown in figure 4.

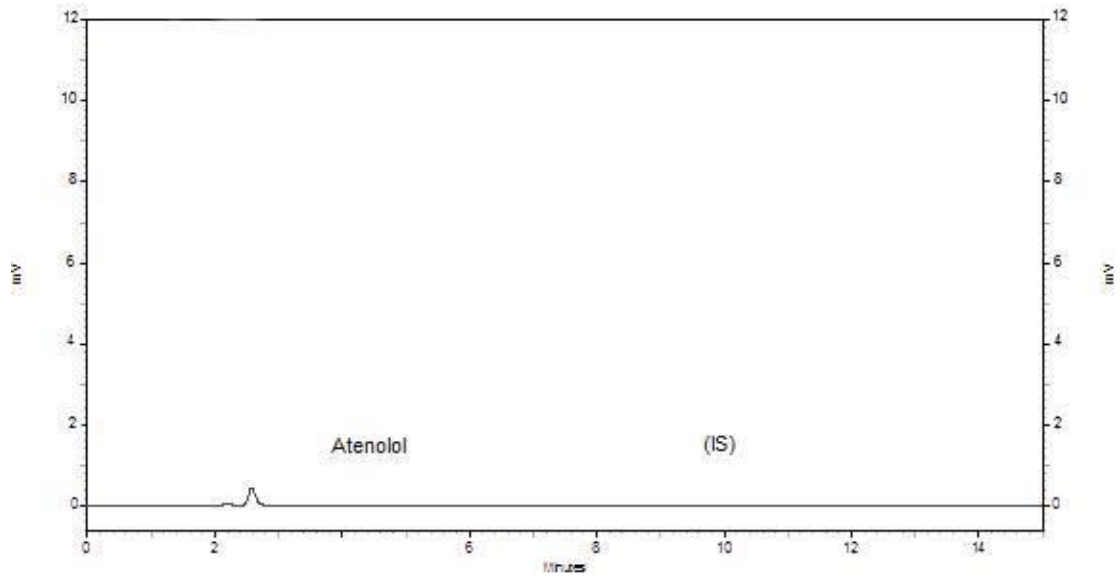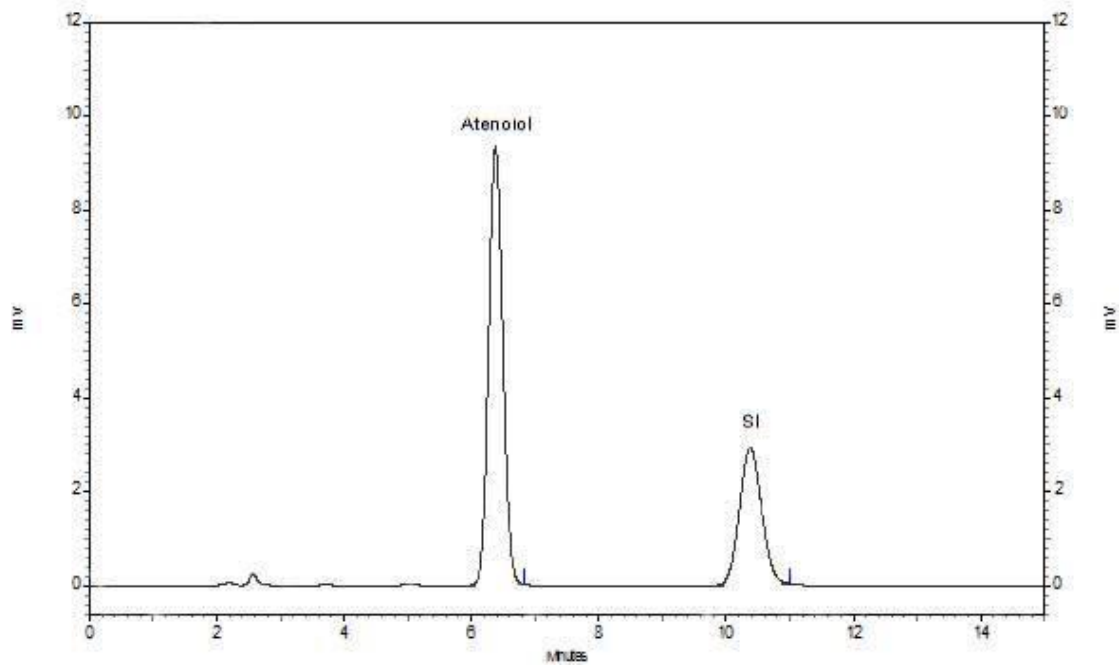**Fig. 3: HPLC chromatogram of a blank human plasma sample.**



**Fig. 4: HPLC chromatogram representing complete resolution of atenolol peak from its internal standard bamethan sulfate peak at a retention time 6.4 and 10.4min, respectively.**

**Accuracy and Precision**

**Intra-day accuracy and precision**

Intra-day accuracy of the method for atenolol ranged from 98.20 % to 101.49%, while the intra-day precision ranged from 1.36 % to 1.91 % at concentrations of 30, 400 and 700 ng/ml. Data are presented in table 1.

**Table 1: Intra-day precision, accuracy and relative error for atenolol determination in spiked human plasma samples.**

|  | Atenolol concentration in human plasma | | |
|---|---|---|---|
|  | **30ng/ml** | **400ng/ml** | **700ng/ml** |
| **Mean** | 30.381 | 405.958 | 687.405 |
| **±SD** | 0.58 | 6.377 | 9.333 |
| **Precisions as CV%** | 1.909 | 1.571 | 1.358 |
| **Accuracy %** | 101.27 | 101.489 | 98.2 |
| **RE%** | 1.271 | 1.489 | 1.799 |

**Inter-day accuracy and precision**

Inter-day precision of the method for atenolol ranged from 1.471 % to 3.246 concentrations 30, 400 and 700 ng/ml. Data are presented in table 2.

**Table 2: Inter-day precision, accuracy and relative error for atenolol determination in spiked human plasma samples.**

|  | Atenolol concentration in human plasma | | |
|---|---|---|---|
|  | **QC        sample 30ng/ml** | **QC        sample 400ng/ml** | **QC        sample 700ng/ml** |
| **Mean** | 29.862 | 402.962 | 702.456 |
| **±SD** | 0.964 | 5.927 | 22.799 |
| **Precisions as CV%** | 3.229 | 1.471 | 3.246 |
| **Accuracy %** | 99.54 | 100.741 | 100.351 |
| **RE%** | 0.46 | 0.741 | 0.351 |

The coefficient of variation% for the all levels of atenolol plasma samples were found to be within acceptable limit indicating a reasonable intermediate precision (intra and inter- day) of the proposed method.

**Accuracy and precision for Quality Control samples**

Quality Control samples were analyzed for atenolol at the three levels. Results are given in table 3.

**Table 3: Accuracy and precision for atenolol quality control samples.**

|  | Atenolol concentration in human plasma | | |
|---|---|---|---|
|  | QC          Low (30ng/ml) | QC Mid (400ng/ml) | QC          High (700ng/ml) |
| Mean | 30.717 | 398.459 | 701.735 |
| ±SD | 1.425 | 13.609 | 6.389 |
| Precisions as CV% | 4.638 | 3.415 | 0.911 |
| Accuracy % | 102.389 | 99.615 | 100.248 |
| RE% | 2.289 | 0.385 | 0.248 |

**Recovery**

The absolute and relative recovery determined for Atenolol shown to be consistent, precise and reproducible at the three levels 30, 400 and 700 ng/ml. Data are depicted in table 4. While, the absolute analytical recovery of Bamethan (as IS) was found to be 81.01%.

**Table 4: Absolute and relative analytical recovery of atenolol**

| Concentration ng/ml | Absolute recovery % | Relative recovery % |
|---|---|---|
| 30 | 83.447 | 100.733 |
| 400 | 87.239 | 99.948 |
| 700 | 79.907 | 98.599 |

**Sensitivity**

The sensitivity of the method was established at 10 ng/ml (lower limit of quantitation), with a precision of 1.754 %. Data for LLOQ is presented in table 5.

**Table 5: Lower limit of quantitation (LLOQ)**

| Concentration (nglml) | Actual concentration (ng/ml) | Accuracy % | RE% | Mean (ng/ml) | CV% |
|---|---|---|---|---|---|
| 10 | 10.042 | 100.42 | 0.42 | 10.163 | 1.754 |
| | 9.939 | 99.39 | 0.61 | | |
| | 10.222 | 102.22 | 2.22 | | |
| | 10.209 | 102.09 | 2.09 | | |
| | 10.401 | 104.01 | 4.01 | | |

**Stability**

*Short-term stability*

The stability of atenolol plasma samples was tested and Calculated data indicate reliable stability behavior under the experimental conditions of the regular run. Results are given below in table 6.

**Table 6: Short-term stability of atenolol in human plasma for five run at different concentration levels.**

| | Short-term stability | | | | | |
|---|---|---|---|---|---|---|
| | Low level (30ng/ml) | | Mid level (400ng/ml) | | High level (700ng/ml) | |
| | Initial analysis conc. (ng/ml) | Analysis after 6hrs, conc. (ng/ml) | Initial analysis conc. (ng/ml) | Analysis after 6hrs, conc. (ng/ml) | Initial analysis conc. (ng/ml) | Analysis after 6hrs, conc. (ng/ml) |
| Mean | 30.791 | 29.001 | 390.699 | 401.055 | 732.325 | 713.715 |

| (ng/ml) | | | | | | |
|---|---|---|---|---|---|---|
| ± SD | 0.699 | 0.891 | 12.515 | 11.388 | 9.741 | 30.146 |
| CV% | 2.269 | 3.072 | 3.203 | 2.839 | 1.33 | 4.224 |
| Recovery % | | 94.186 | | 102.65 | | 97.458 |

*Post-preparative stability*

Stability of samples in the autosampler was assessed. The data is shown in tables 7.

**Table 7: Post-Preparative stability of atenolol in human plasma at different concentration levels**

| | Post-term stability | | | | | |
|---|---|---|---|---|---|---|
| | **Low level (30ng/ml)** | | **Mid level (400ng/ml)** | | **High level (700ng/ml)** | |
| | Initial analysis conc. (ng/ml) | Analysis after 24 hrs, conc. (ng/ml) | Initial analysis conc. (ng/ml) | Analysis after 24 hrs, conc. (ng/ml) | Initial analysis conc. (ng/ml) | Analysis after 24 hrs, conc. (ng/ml) |
| **Mean (ng/ml)** | 30.355 | 30.69 | 398.615 | 401.467 | 711.488 | 723.079 |
| **± SD** | 0.323 | 0.446 | 1.284 | 2.063 | 2.356 | 2.641 |
| **CV%** | 1.064 | 1.455 | 0.322 | 0.514 | 0.331 | 0.365 |
| **Recovery %** | | 101.106 | | 100.715 | | 101.629 |

*Freeze and thaw stability*

The data that represents the stability of Atenolol plasma samples over the cycles of freeze (at -20ºC) and thawing (at room temperature) is given in table 8.

**Table 8: Freeze and thaw stability of atenolol in human plasma at different concentration levels**

| | Freeze and thaw stability | | | | | |
|---|---|---|---|---|---|---|
| | Low level (30ng/ml) | | Mid level (400ng/ml) | | High level (700ng/ml) | |
| | Initial analysis conc. (ng/ml) | Analysis after 3 freeze and thaw cycles, conc. (ng/ml) | Initial analysis conc. (ng/ml) | Analysis after 3 freeze and thaw cycles, conc. (ng/ml) | Initial analysis conc. (ng/ml) | Analysis after 3 freeze and thaw cycles, conc. (ng/ml) |
| Mean (ng/ml) | 31.434 | 29.321 | 392.299 | 404.898 | 730.925 | 719.741 |
| ± SD | 0.429 | 0.859 | 8.231 | 10.555 | 7.962 | 44.746 |
| CV% | 1.366 | 2.929 | 2.098 | 2.606 | 1.089 | 6.217 |
| Recovery % | | 93.275 | | 103.212 | | 98.469 |

*Long- term stability*

The stability data of atenolol in plasma samples stored for a period of eight weeks (beyond that expected for finalizing the authentic samples analysis) at $-20^{o}$C is summarize in table 9.

**Table 9: Long-term stability of atenolol in human plasma at different concentration levels**

| | Long-term stability | | | | | |
|---|---|---|---|---|---|---|
| | Low level (30ng/ml) | | Mid level (400ng/ml) | | High level (700ng/ml) | |
| | Initial analysis conc. (ng/ml) | Analysis after 8 weeks storage, | Initial analysis conc. (ng/ml) | Analysis after 8 weeks storage, | Initial analysis conc. (ng/ml) | Analysis after 8 weeks storage, |

| | | conc. (ng/ml) | | conc. (ng/ml) | | conc. (ng/ml) |
|---|---|---|---|---|---|---|
| Mean (ng/ml) | 30.834 | 29.829 | 392.899 | 397.682 | 728.125 | 695.998 |
| ± SD | 0.617 | 1.333 | 9.802 | 8.508 | 8.218 | 15.371 |
| CV% | 1.999 | 4.469 | 2.495 | 2.139 | 1.128 | 2.208 |
| Recovery% | | 96.739 | | 101.218 | | 95.588 |

The stability study of atenolol in human plasma showed reliable stability behavior, thus suggesting that storage of volunteers' plasma at - 20$^{o}$C is adequate, and no stability-related problems would be expected during the samples routine analysis for the pharmacokinetics studies.

### *Stock solutions stability*

Stability of stock solutions of atenolol and bamethan after 6hrs, expressed as recovery %, was found to be 100.54% and 101.05, respectively.

The stability of stock solutions was tested and established at room temperature for 6 hours. However, results revealed acceptable stability for the prepared stock solutions throughout the period intended for their daily use. Working solutions and serial dilution standard solutions were prepared freshly just before spiking for both the calibration curve and the QC's and were not allowed to stand for a period of time more than that needed to complete spiking of plasma samples.

### Application of the validated method to routine analysis of atenolol

The developed and validated analytical method was utilized for atenolol determination in plasma samples of ten volunteers intended for pharmacokinetics study as shown in table 10.

**Table 10: Plasma concentration (ng/ml) of atenolol after single dose of tenormin$^{®}$ tablets (100 mg) at each time point, n = 10 volunteers.**

| Time (hr.) | Mean (ng/ml) | ± SD | %CV | Min. value | Max. value |
|---|---|---|---|---|---|
| 0.5 | 160.892 | 44.01 | 27.353 | 88.15 | 229.48 |

| | | | | | |
|------|---------|---------|--------|--------|--------|
| 1.0 | 350.218 | 83.551 | 23.857 | 204.65 | 465.11 |
| 1.5 | 456.663 | 105.282 | 23.054 | 231.05 | 620.88 |
| 2.0 | 481.63 | 116.08 | 24.101 | 266.15 | 627.45 |
| 2.5 | 499.14 | 110.345 | 22.107 | 347.06 | 652.13 |
| 3.0 | 520.048 | 109.941 | 21.141 | 349.97 | 683.87 |
| 3.5 | 485.415 | 98.399 | 20.271 | 299.00 | 680.79 |
| 4.0 | 443.874 | 89.876 | 20.248 | 259.28 | 574.18 |
| 6.0 | 313.936 | 79.574 | 25.347 | 176.3 | 426.36 |
| 8.0 | 242.031 | 73.53 | 30.381 | 153.52 | 397.33 |
| 12.0 | 146.833 | 43.67 | 29.741 | 85.67 | 234.46 |
| 16.0 | 92.69 | 27.683 | 29.867 | 55.56 | 142.36 |
| 24.0 | 41.5 | 14.455 | 34.833 | 27.48 | 75.87 |
| 30.0 | 24.943 | 7.647 | 30.658 | 14.7 | 40.23 |
| 36.0 | 16.982 | 1.179 | 6.943 | 15.5 | 18.81 |

Thirty minutes before dosing (at zero time) of tenormin[®] tablets, atenolol was not detected in plasma in all volunteers. The drug was detected in plasma samples of all the 10 volunteers after 0.5 hour of tenormin[®] tablets . This indicate rapid absorption of atenolol from tenormin tablets as reported (3, 4).

The reported value of the mean terminal elimination half-life of atenolol in plasma following the administration of atenolol tablets was about 7 hours (range 4-8 hours). Therefore, blood sampling for 36 hours following Tenormin[®] administration was considered adequate enough for pharmacokinetic study (3, 4).

[Standard Deviation (SD), Coefficient of Variation (CV), Minimum Values (Min), Maximum Values (Max)].

The following pharmacokinetic parameters $C_{max}$ , $t_{max}$ , $AUC_{0-t}$ , $AUC_{t-\infty}$ , $AUC_{0-\infty}$ , $C_{max}/AUC_{0-\infty}$ , , $\beta$ and $t_{0.5}$ were obtained from pharmacokinetic treatment of the individual plasma concentrations vs time data for tenormin[®] tablets (Table 11). The mean of $C_{max}$, $t_{max}$, $AUC_{0-\infty}$ and $t_{0.5}$ value obtained in the present study is comparable to that reported in other studies (6, 18).

The mean value of the % $AUC_{extrapolated}$ ($AUC_{t-\infty}/AUC_{0-\infty} \times 100$) (Residual area) found in this study had small contribution to the total AUC ($AUC_{0-\infty}$) since the

maximum % $AUC_{extrapolated}$ did not exceed 10.5. Thus, indicating an adequate enough sampling program used in the study (13, 17-19).

The ratio $C_{max}/AUC_{0-\infty}$ was calculated as an estimate for the rate of drug absorption (13, 17-19).

The First order terminal elimination rate constant ($\beta$) for tenormin was measured from not less than three points in the terminal phase of the log-concentration vs time data (Tables 10, Fig. 5). This procedure is assumed to give reliable estimation of the $\beta$ value and consequently the values of the pharmacokinetic parameters calculated from $\beta$ value; namely $AUC_{extrapolated}$ and $t_{0.5}$ (13, 17-19).  It was shown that this method is suitable and efficient for the analysis of atenolol in the human plasma samples collected for pharmacokinetic studies.

**Table 11: Pharmacokinetic parameters of atenolol  following administration of a single dose of tenormin® (100mg Tablets) to 10 healthy volunteers.**

| Pharmacokinetic parameters | Mean | ± SD | % CV | Min. value | Max. value |
|---|---|---|---|---|---|
| $C_{max}$ (ng/ml) | 566.596 | 90.615 | 15.992 | 405.35 | 683.87 |
| $t_{max}$ (hr) | 2.7 | 0.752 | 27.88 | 1.5 | 4 |
| $AUC_{0-t}$ (ng.hr/ml) | 5007.04 | 931.205 | 18.597 | 3292 | 6332.6 |
| $AUC_{t-\infty}$ (ng.hr/ml) | 210.791 | 105.176 | 49.896 | 117.95 | 493.97 |
| $AUC_{0-\infty}$ (ng.hr/ml) | 5217.82 | 965.822 | 18.51 | 3494.5 | 6547.4 |
| $\%AUC_{extra}$ | 4.066 | 1.691 | 41.59 | 2.28 | 7.87 |
| $Cmax/AUC_{0-\infty}$ ($hr^{-1}$) | 0.11 | 0.015 | 13.886 | 0.088 | 0.138 |
| $\beta$ ($hr^{-1}$) | 0.094 | 0.015 | 16.325 | 0.078 | 0.0125 |
| $t_{0.5}$ (hr) | 7.562 | 1.106 | 14.633 | 5.56 | 8.94 |
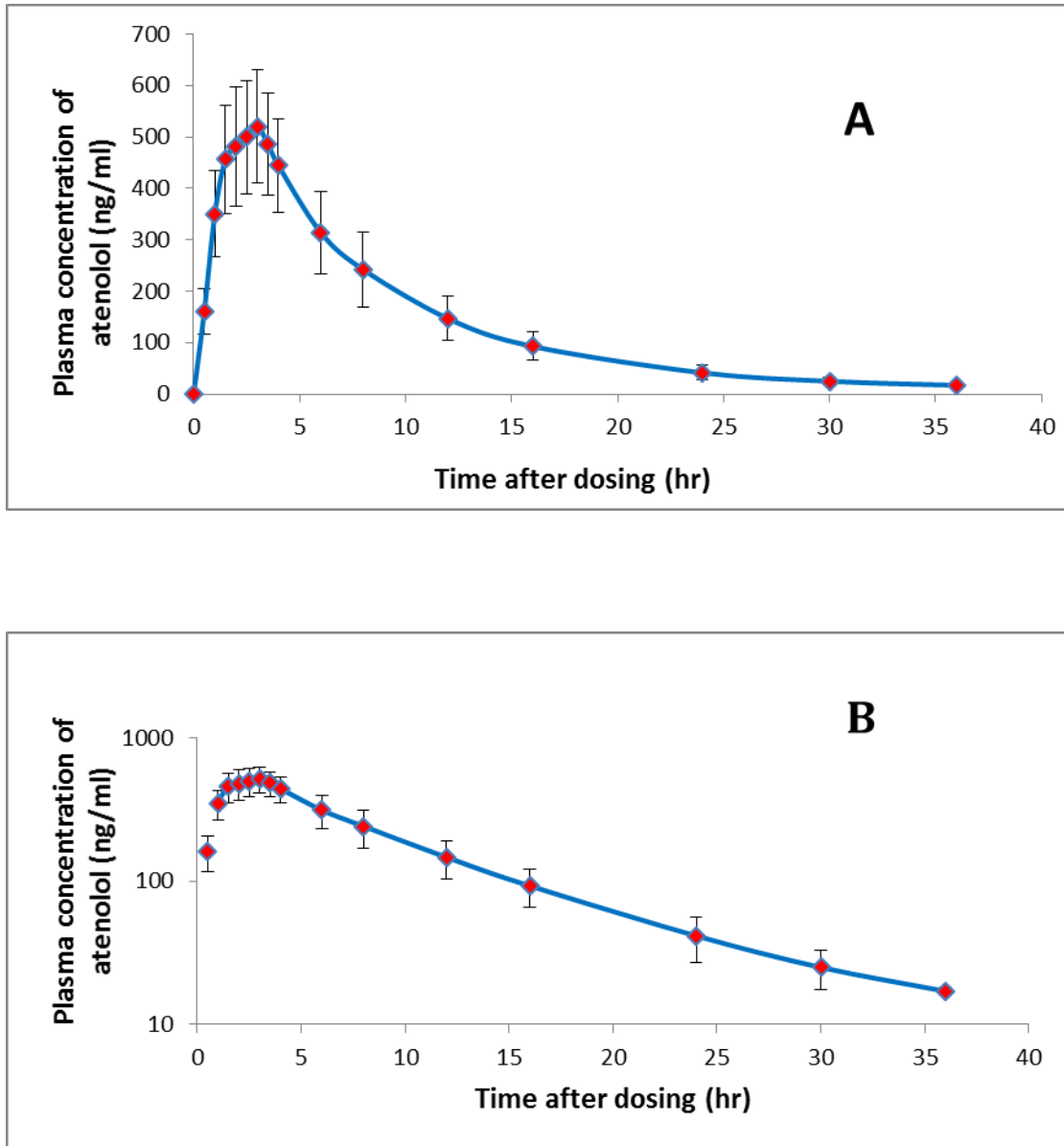
**Figure 5: Plasma concentrations of atenolol (mean ± SD) versus time for ten healthy volunteers plots in rectilinear (A) and in semi-log (B) graph type**

**CONCLUSIONS**

The new developed method of analysis provided a sensitive, precise, accurate and specific assay for atenolol determination in human plasma. One can expect that,

this method would be efficient in analyzing large number of plasma samples; it can be employed for the routine analysis of drug concentrations in bioavailability and bioequivalence studies.

**REFERENCES**

1- The Merck index, an encyclopedia of chemicals, drugs, and biological. 14th ed. Maryadele J.O. Neil, Editor, Merck & Co. INC, USA (2006), p 859.

2- Remington. The science and practice of pharmacy 21th ed., Lippincott Williams and Wilkins (2006), p1401.

3- Martindale. The Complete Drug Reference, 37th ed. Sweetanon, S.C., arm, B.P. Pharms, F.R. London. Chicago, Ph. Press. 2011, P 1290, 1273, 1277, 1294,1340 – 1341.

4- Kirch,W., Gorg, K.G. Clinical pharmacokinetics of atenolol – A review. European Journal of Drug Metabolism and Pharmacokinetics. 1982; 7(2): 81 – 91.

5-Sridharan, D.,  Thenmozhi, A., Sundaranandavalli, S. Bioanalytical method development and validation of atenolol in human plasma by LC-MS. Asian Journal of Pharmaceutical and Clinical Research. 2010; 3(2): 92 – 94.

6- Pires de Abreu, L.R., Calafatti de Castro, S.A., Pedrazzoli, J. Atenolol quantification in human plasma by high-performance liquid chromatography: Application to bioequivalence study. AAPS PharmSci 2003; 5 (2): 116 – 122.

7- Yilmaz, B., Arslan, S., Asci, A. HPLC Method for determination of aenolol in human plasma and application to a pharmacokinetic study in turkey. Journal of Chromatographic Science. 2012; 00:1 – 6.

8- Belal, F., Sharaf El-Din, M., Aly, F., Hefnawy, M., El-Awady, M. Stability-indicating HPLC method for the determination of atenolol in pharmaceutical preparations. J Chromatography Separation Techniques.2013; 4(1): 1 – 7.

9- Parusu, T.,  Ponneri, V. RP-HPLC method for simultaneous determination of atenolol and indapamide in pharmaceutical dosage forms, human blood and milk. European Journal of Chemistry. 2012; 3 (2): 138-142.

10- Elgawish, M.S., Mostafa, S.M., Elshanawane, A.A. Simple and rapid HPLC method for simultaneous determination of atenolol and chlorthalidone in spiked human plasma. Saudi Pharmaceutical Journal. 2011;19 : 43 – 49.

11- Bhusari, V. K., Dhaneshwar S. R. Validated HPLC method for simultaneous quantitation of amlodipine besylate, atenolol and aspirin in bulk drug and formulation. Journal of Pharmaceutical and Bioanalytical Sciences(JPBMS).2012; 17(17):1 – 6 .

12- Mallu, U.R., Pyreddy, V.R., Kota, C.M.R., Kamatham, S., A noval RP-HPLC method for the determination of aspirin, atenolol, atorvastatin and losartan in pharmaceutical drug products. International Journal of Research and Reviews in Pharmacy and  Applied Sciences (IJRRPAS). 2011; 1 (1):1-12.

13- Guidance For Industry. Statistical Approaches to Establishing Bioequivalence. U.S. Department of Health and Human Services. Food and Drug Administration. Center for Drug Evaluation and Research (CDER), January 2001.

14- Guidance for Industry. Bioanalytical Method Validation for human studies. U.S. Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER). May (2001), p 1 – 22.

15- Shabir, G.A. Validation of high- performance liquid chromatography methods for pharmaceutical analysis, understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. Journal of Chromatography A. 2003; 987: 57 – 66.

16- Pathuri, R., Muthukumaran, M.,  Krishnamoorthy, B., Nishat, A. A Review on analytical method development and validation of pharmaceutical technology. Current Pharma Research. 2013;3 (2): 855-870.

17- Shargel, L., Wu-Pong, S., YU, A. Applied Biopharmaceutics & Pharmacokinetics. International edition, 5th ed., The Mc Graw-Hill Companies, Inc. New York, (2005), p 28-37, 45-47, 460-462

18- Mirfazaelian, A., Tabatabaeifar, M., Rezaee, S., Mahmoudian, M. Bioequivalence study of atenolol: Pharmacokinetic and pharmacodynamic evaluation.Journal of DARU 2003; 11(3): 1 – 4.

19- Design And Analysis Of Bioavailability And Bioequivalence Studies. Second Edition, Revised and Expanded. Shein-Chung Chow & Jen-Pei Liu., 2000.