Atenolol Quantification in Human Plasma by High-Performance Liquid Chromatography: Application to Bioequivalence Study

Submitted: August 7, 2002; Accepted: April 18, 2003; Published: June 16, 2003

Luis Renato Pires de Abreu,¹ Silvana Aparecida Calafatti de Castro,² and José Pedrazzoli Jr²

¹Department of Pharmacology, Faculty of Medical Sciences-UNICAMP, Campinas, São Paulo, Brazil

²Integrated Unit of Pharmacology and Gastroenterology-São Francisco University Medical School, Bragança Paulista, São Paulo, Brazil

ABSTRACT

An accurate, precise, and sensitive high-performance liquid chromatography (HPLC) assay was developed for the determination of atenolol in human plasma samples to compare the bioavailability of 2 atenolol tablet (50 mg) formulations in 24 volunteers of both sexes. The study had an open, randomized, 2-period crossover design with a 1-week washout period. Plasma samples were obtained over a 24-hour interval. Atenolol concentrations were analyzed by combined reversed phase liquid chromatography and fluorescence detection ($\lambda_{EX} = 258$ nm, $\lambda_{EM} = 300$ nm). From the atenolol plasma concentration versus time curves, the following pharmacokinetic parameters were obtained: AUC_{0-24h} , $AUC_{0-\infty}$, and C_{max} . The geometric mean of test/reference 50-mg tablets individual percent ratio was 102.2% for AUC_{0-24h} , and 101.6% for C_{max} . The 90% confidence intervals (CI) were 100.2% to 105.4% and 100.9% to 103.5%, respectively. Since the 90% CI for both C_{max} and AUC_{0-24h} were within the 80% to 125% interval proposed by the Food and Drug Administration, it was concluded that atenolol (50-mg tablets) test formulation was bioequivalent to the reference formulation, with regard to both the rate and extent of absorption.

KEYWORDS: atenolol, bioequivalence, HPLC, plasma, determination

INTRODUCTION

Atenolol (CAS 29122-68-7), designated chemically as (RS)-4-(2-hydroxy-3-isopropylaminopropoxy) phenylacetamide, is commercially available as a racemic mixture (rac-atenolol). **Figure 1** shows the structures of atenolol and metoprolol (internal standard). The (–)S-form is the active isomer with no significant pharmacological activity reported for the R(+)-isomer.^{1,2} Atenolol (ATN) is a β_1 -selective (cardioselective) β -adrenergic receptor-blocking agent without membrane-stabilizing or intrinsic sympathomimetic (partial agonist) activities. This preferential effect is not absolute however, and, at higher doses, ATN inhibits β_2 -adrenoreceptors, chiefly located in the bronchial and vascular musculature.³

Like other antihypertensive drugs, ATN lowers the systolic and diastolic blood pressure by 15% to 20% in a single drug treatment. In long-term treatment, it has the ability to reduce cardiovascular mortality.⁴ ATN is also used to treat myocardial infarction (heart attack) and arrhythmias (rhythm disorders), angina (chest pains), and disorders arising from decreased circulation and vascular constriction, including migraine. ATN may be used alone or concomitantly with other antihypertensive agents including thiazide-type diuretics, hydralazine, prazosin, and α -methyldopa.⁵ The most serious adverse effects are heart failure, heart block, and bronchospasm. Reactions tend to be more severe after intravenous injection as opposed to oral administration.⁶ In humans, 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 blood levels are reached between 2 and 4 hours after ingestion. Unlike propranolol or metoprolol, but like nadolol, ATN undergoes little or no metabolism by the liver, and the absorbed portion is eliminated primarily by renal excretion. Over 85% of an intravenous dose is excreted in urine within 24 hours compared with approximately 50% for an oral dose. ATN also differs from propranolol in that only a small amount (6%-16%) is bound to proteins in the plasma. This kinetic profile results in rela-

Corresponding Author: Luis Renato Pires de Abreu, 288 Simao Ferreira Alves St, CEP: 13801-525, PO Box 63, Mogi Mirim, São Paulo, Brazil; Tel: +55 19 38627110; Fax: +55 19 38627110; Email: lrpabreu@uol.com.br.

Figure 1. Structures of atenolol and metoprolol (internal standard).

tively consistent plasma drug levels with about a 4-fold interpatient variation.³⁻⁷ The elimination half-life of oral ATN is approximately 6 to 7 hours, and there is no alteration of the kinetic profile of the drug by chronic administration. Following oral doses of 50 mg or 100 mg, both β -blocking and antihypertensive effects persist for at least 24 hours. When renal function is impaired, elimination of ATN is closely related to the glomerular filtration rate; significant accumulation occurs when the creatinine clearance falls below 35 mL/min/1.73m².^{8,9}

The purpose of this study was to develop an accurate, precise, and sensitive HPLC assay for the determination of ATN in human plasma samples to compare the bioavailability of 2 atenolol tablet (50 mg) formulations in 24 volunteers of both sexes.

MATERIALS AND METHODS

Drugs and Chemicals

Acetonitrile (ACN) and hexane were purchased from EM Science (Gibbstown, NJ); methanol (MeOH), n-butanol, hydrochloric acid, sodium hydroxide, and sodium phosphate salts (Na₂HPO₄/NaH₂PO₄) were purchased from Merck (Rio de Janeiro, RJ, Brazil). Dodecyl sulfate sodium salt (SDS) was purchased from Sigma Chemicals (St Louis, MO). The HPLC-grade solvents were used as received. Atenolol and metoprolol (internal standard) were purchased from USP Pharmacopeia (New York, NY).

Standard Solutions

Stock solution of atenolol was prepared in MeOH (1 mg/mL). Solutions of 1, 10, and 100 μ g/mL were prepared by diluting the stock solution in MeOH. Stock solution of metoprolol tartrate was prepared in MeOH (1 mg/mL). Solutions of 1, 10, and 100 μ g/mL were prepared by diluting the stock solution in MeOH:H₂O (1:1).

Solutions were protected from light and stored at -20°C until used.

Extraction Procedure

To 500 μ L of each standard (ATN-free serum spiked with known amounts of ATN) or test serum sample, 100 μ L of metoprolol of 5 μ g/mL in MeOH/H₂O (1:1) and 200 μ L of NaOH (0.25 mol.L⁻¹) were successively added. After a brief vortex mixing, 3.5 mL of the extraction solvent (hexane:n-butanol, 1:1) was added, and the extraction of the compounds of interest was performed by vortex mixing for 20 seconds. The tubes were then centrifuged (14 000 rpm at 25°C for 10 minutes) and the organic layer transferred to another set of clean tubes to be back extracted with 250 μ L of 0.1 mol.L⁻¹ hydrochloric acid (vortex mixing for 20 seconds). The tubes were then centrifuged (14 000 rpm at 25°C for 10 minutes), the organic layer was discarded, and the aqueous phase was transferred to a clean tube to be evaporated to dryness at 40°C , under an N₂ stream. The residue was dissolved with 100 μ L of the mobile phase, transferred to the injection vials, and a 20- μ L aliquot was injected into the chromatographic system.

Instruments and Chromatographic Conditions

The analyses were performed on a Shimadzu chromatographic system (Kyoto, Japan) equipped with an LC-10AD VP pump, an SIL-10A VP auto-sampler, an RF 535 fluorescence detector, and an SCL-10A VP controller unit. The drug analysis data were acquired and processed with CLASS-VP (v. 6.2) software running under Windows 98 on a Pentium II PC. The mobile phase consisted of a mixture of ACN/MeOH/0.01 mol.L⁻¹ phosphate buffer with pH adjusted to 6.0 with NaOH, containing 0.1% SDS (15:60:25 vol/vol/vol), pumped at a flow rate of 1.2 mL/min through the column (Lichrosorb, 10 μ m RP 18-250 $\times$ 4.6 mm (Merck) with a guard column Security-guard; Phenomenex, CA) at room temperature. Peaks were monitored by fluorescence ($\lambda_{\text{EX}} = 258$ nm, $\lambda_{\text{EM}} = 300$ nm), high sensitivity mode at 23°C . Quantification of ATN was obtained by plotting ATN to internal standard peak height ratios as a function of the concentrations.

Stability

Drug-free plasma was spiked with known amounts of the drug to achieve the concentrations 75, 400, and 700 ng/mL ($n = 3$) and stored at -70°C . These samples were used to investigate the stability of ATN over a period of 1 month. No internal standard was added prior to the analysis.

Specificity

The specificity of the method was determined by comparing the chromatograms obtained from the samples containing ATN and internal standard with those obtained from blank samples.

Limit of Detection and Limit of Quantification

Limit of detection (LOD) is a parameter that provides the lowest concentration of analyte in a sample that can be detected, but not quantified, under the stated experimental conditions. LOD was determined using the signal-to-noise ratio by comparing test results from samples with known concentrations of analyte to blank samples. The analyte concentration that produced a signal-to-noise ratio of 3:1 was accepted as the LOD. Limit of quantification (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions.

Recovery and Linearity

The analytical recovery of ATN was determined at concentrations of 75, 400, and 800 ng/mL ($n = 3$). Drug-free plasma was spiked with known amounts of the drug to achieve the concentration previously specified. These samples were processed by the analytical method described above, and peak heights were compared with the peak heights obtained by direct injection of the drugs in the mobile phase. The linearity study was carried out in the range of 25 to 800 ng/mL. To access linearity, drug-free plasma was spiked with known amounts of the drug to achieve the concentrations of 25, 50, 100, 300, 500, and 800 ng/mL.

Precision and Accuracy

The precision and accuracy data were obtained by analyzing 3 aliquots of spiked plasma at low (75 ng/mL), middle (400 ng/mL), and high (700 ng/mL) concentration levels of ATN. Intra-day reproducibility was determined by analyzing 5 aliquots of spiked human plasma and inter-day reproducibility was determined over a 5-day period ($n = 5$).

Formulations

The following test atenolol formulation was employed: atenolol tablets, 50 mg from Brazil (batch number, 110/00; production date, 09/2000; expiration date,

05/2005). The details of the reference atenolol formulation are as follows: atenol tablets, 50 mg produced by Zeneca do Brasil LTDA (batch number, 1410; production date, 05/2000; expiration date, 05/2004). An in vitro comparative study between the 2 formulations was also performed. Both products meet the United States Pharmacopeia (USP) 23 specification.

Clinical Protocol

Twenty-four adult volunteers, aged 21 to 47 years, weighing 55 to 95 kg, and within 15% of ideal body weight were selected for the study. The volunteers were free from significant cardiac, hepatic, renal, pulmonary, gastrointestinal, neurological, and hematological disease as determined within 4 weeks of the beginning of the study by medical history, physical examination, and laboratory screenings including fasting blood glucose, urea, creatinine, Serum Glutamic Oxaloacetic Transaminase (SGOT) or Aspartate Aminotransferase (AST), Serum Glutamic Pyruvic Transaminase (SGPT) or Alanine Aminotransferase (ALT), total bilirubin, total protein, serum albumin, alkaline phosphatase, sodium, potassium, chloride, uric acid, urinalysis, hemoglobin, hematocrit, and total differential white blood cells count. All volunteers gave written informed consent and the Ethics Committee of São Francisco University Hospital approved the clinical protocol.

The study had an open, randomized, 2-period crossover design with a 7-day washout period between doses. During each period the volunteers were hospitalized, had a regular meal, and received a 50-mg tablet of the ATN, allocated according to the appropriate randomized dose randomization code. After administration of the tablet, the volunteers were asked to drink 200 mL of tap water. Blood samples for serum drug assay were taken from a forearm vein before and at 0, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 18, and 24 hours after ATN administration. On each occasion, one 5-mL sample was taken via "butterfly" into a clean tube. After blood clotting at room temperature, the blood samples were centrifuged at 2000g for 10 minutes, and the plasma was removed and stored at -20°C until assayed. The volunteers received 200 mL of tap water to drink 3 hours after dosing. Six hours after dose administration, a standard lunch was available, and an evening meal was provided 12 hours after dosing. Liquids were allowed ad libitum after lunch, but xanthine-containing beverages, such as tea, coffee, and cola were not permitted.

Pharmacokinetic and Statistical Analysis

Maximum observed plasma concentration (C_{max}) and time taken to reach it (T_{max}) were obtained from drug concentra-

tion versus time curves. The areas under the ATN concentrations versus time curves from 0 to 24 hours (AUC_{0-24h}) were calculated by using the trapezoidal method, and the first order elimination rate constant (K_e) was estimated by the least square regression of the points describing the terminal log-linear decaying phase. $T_{1/2}$ were derived from K_e ($T_{1/2} = \ln 2 / K_e$). The results are expressed as mean $\pm$ SEM. C_{max} and AUC_{0-24h} data were analyzed statistically using both parametric (1-way analysis of variance [ANOVA]) and nonparametric methods (Wilcoxon signed rank test).

RESULTS AND DISCUSSION

The alternative HPLC method described here is similar to other previously published methodologies.⁹⁻¹⁴ However, these methods require the use of relatively large volumes of plasma and extraction solvent, as well as high retention times (about 15 minutes). Other more complicated extraction procedures such as solid-phase extraction were also reported. Nevertheless, solid-phase extraction procedures are laborious and require solid-phase extraction cartridges, increasing the cost of the analysis. In a recent study, Li et al reported the determination of atenolol using LC-MS-MS detection, but mass spectrometers are expensive and not readily available.¹⁵ The method applied here was accurate, precise, sensitive, and capable of determining concentrations of atenolol in small volumes of human plasma. The extraction procedure was efficient, and a commercially available internal standard (metoprolol) was employed. The alternative method proposed presents advantages such as smaller sample and extraction solvent volumes. This assay method was also rapid, once the preparation of 30 samples took less than 1 hour from initial extraction step to final placement of samples in the HPLC auto-sampler vials. Furthermore, the chromatographic run time was 8 minutes, less than the 15 minutes described by Martins et al.¹³

In order to achieve the best selectivity and sensitivity, different extraction procedures were tested. Initially silica-based and C18 solid-phase extraction cartridges were used, resulting in clean chromatograms. Unfortunately, solid-phase extraction procedures were not reproducible. Consequently, ACN and MeOH were used for denaturing of plasma proteins, but no clean blank chromatograms were obtained. Denaturation with acids reduced the assay extraction ratio, perhaps due to decomposition of the drug. Liquid phase extraction methods using different organic solvents were also tried, and the method using the mixture hexane:n-butanol (1:1) yielded the best results, showing good extraction efficiency. However, because of the high extraction capacity of this solvent mixture, no

clean blank chromatograms were obtained. Then, an extra back-extraction step (with hydrochloric acid) was tried and clean blank chromatograms showing good extraction efficiency (>95%) were finally obtained. The addition of the back-extraction step resulted in the elimination of endogenous compounds, improving the LOQ and preventing the blockade of the precolumn. This improvement permitted processing of samples from at least 3 volunteers a day and was successfully applied to about 700 samples collected for a bioequivalence study. Because of high sensitivity and a 500 μ L sample requirement, this method can be used in pharmacokinetics, bioavailability, and bioequivalence studies.

Figure 2 shows that under described chromatographic conditions, the retention times for ATN and internal standard were 3.1 and 6.0 minutes, respectively. As also shown in **Figure 2**, no endogenous interfering peaks appeared at the retention times of the compounds of interest.

Figure 2. Chromatographic analysis of atenolol: (A) plasma blank; (B) plasma + atenolol (700 ng/mL)-1, and internal standard (metoprolol 500 ng/mL)-2; (C) chromatogram obtained from plasma of a volunteer following oral administration of atenolol (50 mg): atenolol (63.4 ng/mL)-1, and internal standard (metoprolol 500 ng/mL)-2.

Figure 3. Calibration curve of atenolol.

Table 1. Analytical Precision and Accuracy of the Determination of Atenolol from Spiked Plasma Samples*

Concentration Added (ng/mL)	Concentration Obtained (ng/mL)	CV (%)	Relative error (%)
<i>Intra-day</i>			
75	67.41	6.2	89.9
400	401.93	8.9	100.5
700	655.18	7.3	93.6
<i>Inter-day</i>			
75	69.37	4.7	92.5
400	373.10	2.9	93.2
700	633.15	5.3	90.4

The mean absolute recovery of ATN in plasma was 98.3%. The LOQ for ATN was 25 ng/mL and the LOD was set at a concentration of 10 ng/mL. The calibration curve was linear over the range 25 ng/mL to 800 ng/mL, with a regression coefficient ≥ 0.99 and an intercept not significantly different from zero (**Figure 3**).

The obtained analytical precision and accuracy for intra-day and inter-day assays of 4 quality controls (25, 75, 400, and 700 ng/mL) are shown in **Table 1**. The overall variability of the quality controls (75, 400, and 700 ng/mL; $n = 48$) was 7.8%, 8.3%, and 8.3%, respectively; the accuracy was 95.9%, 99.0%, and 92.5%, respectively. No significant degradation of ATN was observed during this period under the storage conditions.

ATN was well tolerated at the administered dose, and no adverse effects were reported. **Figure 4** shows mean ATN plasma concentrations as a function of time after the oral administration of 50 mg ATN of both brands.

The major mean pharmacokinetic parameters derived from the serum concentration versus time curves are presented in **Table 2**.

Geometric means ratios for AUC_{0-24h} and C_{max} of the 2 ATN oral formulations are shown in **Table 3**, as are the analyses of their inclusion into the interval 80% to 125% by different tests.

Figure 4. Curves of the mean plasma concentration of atenolol ($\pm$ SEM) of 24 volunteers vs time (h).

Table 2. Mean Pharmacokinetic Parameters Obtained in 24 Healthy Volunteers After the Administration of Both Atenolol 50-mg Formulations*

Parameters	(Test)	(Standard)
AUC_{0-24h} ($\mu\text{g.h.mL}^{-1}$)		
Geometric mean	1.81	1.56
(CI 90%)	(1.6-2.0)	(1.33-1.80)
$AUC_{0-\infty}$ ($\mu\text{g.h.mL}^{-1}$)		
Geometric mean	2.13	1.77
(CI 90%)	(1.83-2.44)	(1.54-2.00)
C_{max} ($\mu\text{g.mL}^{-1}$)		
Geometric mean	0.33	0.30
(CI 90%)	(0.29-0.37)	(0.24-0.35)
$t_{1/2}$ (h)		
Geometric mean	5.07	5.65
(CI 90%)	(3.82-6.31)	(2.56-8.74)
T_{max} (h)		
Geometric mean	2.74	2.64
(CI 90%)	(2.29-2.99)	(2.08-3.20)

* CI indicates Confidence Interval.

Table 3. Statistical Analysis of AUC_{0-24h} and C_{max} Ratios Between Atenolol (Test) and Atenol (Standard)*

Test/Standard % Ratio	AUC _{0-24h}	C _{MAX}
Geometric Mean	102.19	101.61
90% CI	(100.23-105.41)	(100.94-103.51)

*CI indicates Confidence Interval.

CONCLUSION

The validated HPLC method employed here proved to be simple, fast, reliable, selective, and sensitive enough to be used in clinical pharmacokinetic studies of ATN in humans. The median T_{max} and C_{max} values for both oral formulations were similar to those reported in the literature. The present study shows that atenolol 50-mg tablets was bioequivalent to Atenol tablets 50 mg, with regard to both the rate and extent of absorption, as assessed by the inclusion of 90% CI for both AUC_{0-24h} and C_{max} ratios into the interval 80% to 125% proposed by the European Union and US Food and Drug Administration.¹⁶⁻¹⁹

REFERENCES

- Clementi WA, Garvey TQ, Clifton GD, McCoy RA, Brandt S, Schwartz S. Single dose pharmacokinetics of (S)-atenolol administered orally as a single enantiomer formulation and as a racemic mixture (Tenormin). *Chirality*. 1994;6(3):169-174.
- Egginger G, Lindner W, Karh S, Stoschitzky K. Stereoselective HPLC bioanalysis of atenolol enantiomers in plasma: application to a comparative human pharmacokinetic study. *Chirality*. 1993; 5: 506-512.
- Physician's Desk Reference-PDR, 51st ed, Montvale, NJ: Medical Economics Company, Inc; 1999:1548.
- Cruickshank JM, McAlinsh J. Atenolol and ischemic heart disease: an overview. *Curr Med Res Opin*. 1991;12:485-496.
- Wadworth AN, Murdoch D, Brogden RN. Atenolol: a reappraisal of its pharmacological properties and therapeutic use in cardiovascular disorders. *Drugs*. 1991;42:468-510.
- Psaty BM, Koepsell TD, LoGerfo JP, et al. Beta-blockers and primary prevention of coronary heart disease in patients with high blood pressure. *JAMA*. 1989;261:2087-2094.
- Heel RC, Brogden RN, Speight TM, Avery GS. Atenolol: a review of its pharmacological properties and therapeutic efficacy in angina pectoris and hypertension. *Drugs*. 1979;17:425-460.
- Majid PA, van der Vijgh WJF, Waddeh R, van der Wall EE, Roos JP. Once daily atenolol (Tenormine) in the treatment of angina pectoris. *Eur J Cardiol*. 1979; 9(6):419-35.
- Sitzler G, Heibel B, Luckner PW, Palm D. Bioequivalence of two atenolol formulations in healthy volunteers. Evaluation and prediction of effect kinetics at β -adrenoceptors in vivo by means of a radioreceptor assay. *Methods Find Exp Clin Pharmacol*. 1991;13(2):129-137.
- Yee YG, Ruben P, Blaschke TF. Atenolol determination by high-performance liquid chromatography and fluorescence detection. *J Chromatogr*. 1993;171:357-362.
- Shen J, Wanwimolruk S, Hung C T, Zoest AR. Quantitative analysis of beta-blockers in human plasma by reversed-phase ion-pair high-performance liquid chromatography using a micropore column. *J Liq chromatogr*. 1991;14:777-793.
- Terry S, Teitelbaum Z. A liquid chromatographic method for determination of atenolol in human plasma. *J Liq Chromatogr*. 1991;1:3735-3744.
- Martins ML, Pierossi MA, Moraes LA, et al. Comparative bioavailability of two atenolol tablet formulations after a single dose administration. *Int J Clin Pharm Ther*. 1997;35(8):324-328.
- Marzo A, Monti NC, Dal Bo L, et al. Comparative bioavailability of two formulations containing atenolol and chlortalidone associated in a 4:1 fixed combination. *Arzneimittelforschung*. 2000;50(9):802-808.
- Li J, Anari R, Kwok D. Rapid and simultaneous analysis of seven beta-blockers in human plasma using electrospray LC/MS/MS. *AAPS PharmSci*. 1999;1(4). Abstract 2854.
- Food and Drug Administration. Bioavailability and bioequivalence requirements. *Fed Regist*. 1985;320:154-173.
- Food and Drug Administration. Bioavailability and bioequivalence requirements; abbreviated applications; proposed revisions. Proposed rule. *Fed Regist*. 1998;63:64222-64228.
- Food and Drug Administration. Guidance for industry, bioanalytical methods validation for human studies. Food and Drug Administration Web site. Available at: <http://www.fda.gov/cder/guidance/>, 1998b. August/1998.
- Food and Drug Administration. In vivo bioequivalence guidances. *Pharmacopeial Forum*. 1993;19:6501-6508.