Introduction

Atenolol is a cardioselective β1-adrenergic blocker widely used for the treatment of hypertension, angina pectoris and cardiac arrhythmias. A simple, specific, sensitive, precise and accurate high-performance liquid chromatography method with fluorescence detection has been developed and validated for the determination of atenolol in human plasma. After addition of the internal standard, the analytes were extracted by liquid–liquid extraction. The calibration graph for atenolol was linear in a 10–1,000 ng/mL concentration range (r > 0.999), using 0.5-mL plasma samples. The assay precision of the method was less than 6.4%, the assay accuracy ranged between 99.6% and 101.5%, and the absolute recovery of atenolol and internal standard was better than 66.1% and 76.2%, respectively. The method was found to be suitable for the quantification of atenolol in a pharmacokinetic study after a single oral administration of 100 mg atenolol to 18 healthy subjects.

Experimental

Chemicals and reagents

HPLC-grade acetonitrile was obtained from J.T. Baker (Deventer, The Netherlands). Atenolol (assay ≥ 98%) and procainamide hydrochloride (assay ≥ 98%) (internal standard) were purchased from Sigma (St. Louis, MO). Their chemical structures are given in Figure 1. Other chemicals, including analytical grade 1-butanol, methanol, sodium hydroxide, 85% phosphoric acid, 1-octanesulfonic acid sodium salt, triethylamine and n-hexane were obtained from Merck (Darmstadt, Germany). Blank human plasma was obtained from the Blood Donation Unit (AHEPA University Hospital, Thessaloniki, Greece). All other chemicals and solvents were of analytical grade and water was milli-Q grade.

Instrumentation

The development and validation work was carried out on an HPLC system consisting of aISCO 2300 pump (Lincoln, NE), a Perkin-Elmer LS-40 fluorescence detector (Perkin-Elmer, Cambridge, MA), a Hewlett-Packard HP3396A integrator (Avondale, PA) and a Hitachi 655A-40 autosampler (Tokyo, Japan).

Chromatographic conditions

The chromatographic separation was performed on a Brownlee cartridge Spheri-5 cyan analytical column (100 × 4.6 mm i.d.,
5 μm particle size) (Perkin-Elmer). The mobile phase consisted of a mixture of an aqueous solution containing 5 mM triethylam- 
mine, 10 mM phosphoric acid and 7 mM 1-octanesulfonic acid 
sodium salt (pH 2.9) with acetonitrile (60/40, v/v) and was 
filtered through a 0.45-μm pore size nylon filter (Alltech, 
Deerfield, IL) and degassed by ultrasonic treatment before use. 
The HPLC system was operated isocratically at a flow rate of 
0.5 mL/min; the injection volume was 50 μL and the chromato-
graphic peaks were measured by fluorescence detection at 229 
and 309 nm for lex and lem, respectively. Quantitation of aten-
olol was determined by peak height ratio comparisons with 
standard curves, which were prepared daily using drug-free 
plasma samples.

**Quality control samples**

Volumes of 25 mL of human plasma were spiked with appropri-
ate amounts of atenolol stock solutions to obtain quality 
control (QC) samples containing 50, 100 and 500 ng/mL of 
atenolol. These samples were divided into aliquots of approxi-
ately 1 mL into one-dram vials capped tightly and placed at 
−20°C, pending analysis. These samples were used in the anal-
ysis of plasma samples as QCs for the purpose of checking pre-
cision, accuracy and absolute recovery of atenolol in the daily 
analyses of plasma samples.

**Sample preparation**

Plasma samples (0.5 mL) in 13 × 100-mm glass test tubes were 
spiked with 50 μL of calibration standards of atenolol, 25 μL of 
the internal standard, procainamide, and 25 μL of 10M sodium 
hydroxide solution. Liquid–liquid extraction of atenolol and in-
ternal standard was conducted by the addition of 3 mL of 

n-butanol–n-hexane (1:1), followed by vortex at high speed for 
30 s. After centrifugation at 3,000 rpm for 5 min, the upper 
organic phase was transferred into a second 13 × 100-mm glass 
test tube, and after the addition of 1 mL of 0.01% phosphoric 
acid, the mixture was vortexed for 30 s and then was centri-
fuged at 3,000 rpm for 30 s. After aspiration of the upper 
organic phase, the lower aqueous phase was transferred into 
the injection vial and a 50-μL aliquot was injected into the 
chromatographic system for quantitation.

**Assay validation**

The validation of the method was based on the guidelines of 
United States Food and Drug Administration (FDA) (22). The 
assay was validated by assaying seven calibration standards and 
three QC samples in triplicate on three separate occasions. 
Data were obtained through linear regression analysis of peak 
height ratios of atenolol–internal standard (y) versus atenolol 
concentrations (ng/mL) in spiked plasma samples (x). A 
weighting factor of 1/concentration was employed.

Assay accuracy and precision were determined in conjunc-
tion with the linearity studies by assaying on three separate 
ocasions using three QC samples at each of the three concen-
trations (50, 100 and 500 ng/mL). Concentrations of atenolol 
in QC samples were determined by application of the appropri-
ate standard curve obtained on that occasion. Assay accuracy 
was assessed by calculating the estimated concentrations as a 
percent of the nominal concentrations and relative standard de-
viation (RSD) was used to express assay precision. The lower 
limit of quantification (LOLO) was defined as the lowest quanti-
fiable concentration on the calibration curve at which both ac-
curacy and precision should be within 20%.

The absolute recovery of atenolol and internal standard was 
assessed by direct comparison of peak heights from extracted 
versus non-extracted samples by using six replicate plasma 
samples at each of the three atenolol concentrations, 50, 100 
and 500 ng/mL, plus the appropriate amount of internal 
standard.

System reproducibility was assayed by processing nine repli-
cate human plasma samples containing 50, 100 and 500 ng/mL 
atenolol plus internal standard. The final extracts were pooled 
in each case and transferred into nine autosampler vials, and 
the samples were injected into the HPLC.

Freeze–thaw stability of atenolol plasma samples was deter-
mimed by subjecting six aliquots of two QC samples of low and 
high concentration (50 and 500 ng/mL) to three consecutive 
freeze–thaw cycles and comparing the concentrations with 
those of freshly thawed QC samples. Short-term stability of 
atenolol plasma samples at room temperature was determined 
by subjecting six aliquots of two QC samples of low and high 
concentration (50 and 500 ng/mL) to room temperature for 
24 h and comparing the concentrations with those of freshly 
thawed QC samples. An error of less than 10% from the 
expected concentrations for the test QC samples was consid-
ered to be acceptable for both stability tests.

**Pharmacokinetic study**

The developed HPLC method was used to investigate the 
plasma profile of atenolol after a single 100-mg oral dose of an

**Figure 1. Chemical structures of atenolol and procainamide (internal standard).**
atenolol oral formulation. A clinical study on eighteen healthy volunteers was conducted under fasting conditions. Approval to conduct the study was obtained by an independent ethics committee (Clinic “Evangelismos,” Thessaloniki, Greece). Following written informed consent, volunteers received a 100-mg oral dose of an atenolol tablet with 240 mL of water. Blood samples (6 mL) were collected into heparinized test tubes, immediately before (0) and at 20, 40, 60, 90, 120, 150, 180, 240, 300, 360, 480, 600, 720, 960, 1,440 and 2,160 min post-dose. The blood was centrifuged, and the plasma fraction was immediately separated by centrifugation at 3,000 g and stored in polypropylene tubes at −20°C, pending analysis.

Pharmacokinetic analysis was performed using model-independent methods (23). Pharmacokinetic calculations were carried out with the EquivTest/PK software (Statistical Solutions, Cork, Ireland) run on a personal computer. The maximum plasma concentration (Cmax) value and the corresponding time that the latter is marked (Tmax) were taken directly from the individual plasma data. The elimination rate constant (k) was obtained by means of linear regression analysis of the semilogarithmic plasma concentration–time curve and the elimination half-life (t1/2) was calculated by dividing ln2 by k. The area under the plasma concentration–time curve from administration to the last observed concentration at time t (AUC0–t) was estimated by the use of the linear trapezoidal method, according to the following equation:

\[
AUC_{0–t} = \frac{1}{2} \sum_{i=0}^{n-1} \left( t_{i+1} - t_i \right) (C_i + C_{i+1})
\]

where \( t_i \) is the \( i^{th} \) time point, \( C_i \) is the \( i^{th} \) available concentration and \( n \) is the number of data points. The area under the plasma concentration–time curve extrapolated to infinite time (AUC0–\( \infty \)) was estimated by the use of the linear trapezoidal method, according to the following equation:

\[
AUC_{0–\infty} = AUC_{0–t} + \frac{C_n}{k}
\]

where \( C_n \) is the last measurable concentration.

Results and Discussion

**Chromatography**

To obtain the best chromatographic separation and sensitivity in a short time, different mixtures of acetonitrile and aqueous solution containing different additives (e.g., acids, ion pairing reagents and triethylamine), different analytical columns (e.g., C8, C18 and cyano), and different internal standards (e.g., other β-blockers), were systematically investigated. The best separation was achieved with a cyano analytical column (100 × 4.6 mm i.d., 5 μm particle size) and a mobile phase consisting of an aqueous solution containing 5 mM triethylamine, 10 mM phosphoric acid and 7 mM 1-octanesulfonic acid sodium salt with acetonitrile (60/40, v/v). Symmetrical peaks were observed for atenolol and internal standard and the corresponding tailing factors were found to be 1.08 and 1.06, respectively. No endogenous interference with atenolol or internal standard was observed in six different sources of blank plasma. Typical chromatograms obtained with a drug-free plasma and a plasma sample containing 98.7 ng/mL of atenolol and 1.25 μg/mL of internal standard obtained from a volunteer 16 h post-dose, after a single oral dose of 100 mg atenolol, are illustrated in Figure 2. No peaks interfered with atenolol or internal standard. Peak purity for atenolol and internal standard was verifying using HPLC with diode array detection (DAD). More than 800 injections of spiked plasma samples have been made on a single analytical column with minimal loss of chromatographic integrity.

**Extraction procedure**

Many of the previously published HPLC methods for the determination of atenolol in human plasma using solid-phase or liquid–liquid extraction were tested without success, due to many interfering peaks that co-extracted from plasma and interfere with atenolol and internal standard, causing problems with quantification at low concentrations (6, 9, 11, 13). These results led us to investigate other liquid–liquid extractions for

![Figure 2](image-url)
sample pretreatment. The extraction solvent was optimized by examining different solvent systems such as methyl-tert butyl ether, ethyl acetate, either alone or as mixture with n-hexane (1:1), dichloromethane, either alone or as mixture with 2-propanol (3:1), and a mixture of chloroform with 2-propanol (4:1). Unfortunately, the tested extraction solvents produced unclean chromatograms with interfering peaks at the retention times of atenolol and internal standard or low recoveries were observed. Due to the basic character of both atenolol and procaïnamide, pH values 9.6 and 9.32, respectively, before extraction plasma samples were alkalinized (pH 12) with sodium hydroxide. The developed method finally involved the extraction of alkalinized plasma samples with n-butanol–n-hexane (1:1) and back-extraction into acidic aqueous phase, which gave clean chromatograms without interfering peaks at the retention times of atenolol or procaïnamide, the internal standard.

**Assay validation**

The linearity of the method was demonstrated over the concentration range of 10 to 1,000 ng/mL, using 0.5-mL plasma samples. A typical calibration curve had the regression equation of \( y = 0.01877 + 0.02357x \) \( (r = 0.999) \). The assay precision was less than 6.4% based on RSD values of 6.4%, 3.7% and 3.8% for samples containing 50, 100 and 500 ng/mL, respectively. Assay accuracy was ranged between 99.6% and 101.6% (Table 1). The LLOQ was deemed to be 10 ng/mL with both calculated accuracy and precision below 15% and a signal-to-noise (S/N) ratio of 10:1. The LLOQ was sufficient to determine atenolol concentrations in human plasma samples obtained in the conduct of pharmacokinetic studies. The mean absolute recoveries for atenolol were 66.1% ± 2.1%, 67.6% ± 1.2% and 67.2% ± 2.1% at the 50, 100 and 500 ng/mL concentrations, respectively \( (n = 6) \). The mean recovery of the internal standard was found to be 76.2 ± 1.4% \( (n = 18) \). System reproducibility, expressed as %RSD, was 6.2%, 1.3% and 1.0% for atenolol at concentrations of 50, 100 and 500 ng/mL, respectively. System reproducibility for the internal standard was 0.8%. Individual specificity in relation to endogenous plasma components was demonstrated by analysis of a series of randomly selected drug-free samples \( (n = 10) \). Stability studies showed plasma QC samples for atenolol to be stable through all three freeze–thaw cycles and at room temperature for 24 h, with errors better than 2.23% and 2.47%, respectively.

**Application to a pharmacokinetic study**

The method was successfully applied to perform the quantitative determination of plasma concentrations of atenolol after oral administration of a 100-mg dose of an atenolol formulation to 18 healthy volunteers. The mean ± SD plasma atenolol concentration–time data provided the following pharmacokinetic parameters (mean ± SD): \( \text{AU}_{0-\infty} 5,440.1 \pm 1,131.0 \text{ ng.h/mL, } \text{AUC}_{0-1} 5,234.2 \pm 1,147.7 \text{ ng.h/mL, } \text{C}_{\text{max}} 537.1 \pm 112.7 \text{ ng/mL, } T_{\text{max}} 3.4 \pm 1.0 \text{ h and } t_{1/2} 6.7 \pm 1.2 \text{ h.} \) The observed values of the pharmacokinetic parameters were comparable to those reported for atenolol in previous studies (24).

**Conclusions**

A specific, selective, sensitive, precise and accurate HPLC assay with fluorescence detection was developed and validated for the determination of atenolol in human plasma and was found to be suitable for the analysis of large numbers of plasma samples. The assay was used for the analysis of plasma samples obtained from 18 healthy subjects in a pharmacokinetic study, following oral administration of 100 mg of atenolol.

**References**


---

**Table I**

Assay Accuracy and Precision for Atenolol in Quality Control Samples in Human Plasma \( (n = 9) \)

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Accuracy* (%)</th>
<th>Precision (RSD, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>101.6 ± 6.5</td>
<td>6.4</td>
</tr>
<tr>
<td>100</td>
<td>101.0 ± 3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>500</td>
<td>99.6 ± 3.7</td>
<td>3.8</td>
</tr>
</tbody>
</table>

*Accuracy: found concentration expressed as percentage of the nominal concentration.


13. Miller, L.G., Greenblatt, D.J.; Determination of atenolol in plasma by high-performance liquid chromatography with application to single-dose pharmacokinetics; *Journal of Chromatography* (1986); 381: 201–204.


