HPLC Method for Determination of Atenolol in Human Plasma and Application to a Pharmacokinetic Study in Turkey

Bilal Yilmaz[1]*, Sakir Arslan[2] and Ali Asci[3]

[1]Department of Analytical Chemistry, Faculty of Pharmacy, Ataturk University, 25240, Erzurum, Turkey, [2]Department of Cardiology, Faculty of Medicine, Ataturk University, 25240, Erzurum, Turkey, and [3]Department of Toxicology, Faculty of Pharmacy, Ataturk University, 25240, Erzurum, Turkey

*Author to whom correspondence should be addressed. Email: yilmazb@atauni.edu.tr

This paper describes a high-performance liquid chromatography method for the determination of atenolol in human plasma. Atenolol and the internal standard, metoprolol, were extracted from plasma by using a liquid–liquid extraction method. The method was developed on an Ace C18 reverse-phase column using a mobile phase of methanol–water (50:50, v/v) containing 0.1% trifluoroacetic acid. The calibration curve was linear within the concentration range of 5–150 ng/mL. Intra-day and inter-day precision values for atenolol in plasma were less than 6.1, and accuracy (relative error) was better than 5.5%. The mean recovery of atenolol was 98.4% for plasma. The limits of detection and quantification of atenolol were 1.5 and 5 ng/mL, respectively. Also, this assay was successfully applied to six patients with hypertension who had been given an oral tablet of 50 mg atenolol.

Introduction

β-blockers constitute one of the most frequently prescribed groups of cardiovascular drugs. They are used in the management of cardiovascular disorders, such as hypertension, angina pectoris, cardiac arrhythmias and myocardial infarction (1).

Atenolol, 4-(2-hydroxy-3-isopropyl-aminopropoxy) phenylacetamide, is a cardioselective β-blocker (Figure 1). It may be used alone or concomitantly with other antihypertensive agents including thiazide-type diuretics, hydralazine, prazosin and α-methyldopa (2).

Several methods have been reported for the determination of atenolol in human plasma and other biological fluids, including high-performance liquid chromatography (HPLC) (2–8), liquid chromatography–tandem mass spectrometry (LC–MS-MS) (9) capillary electrophoresis (CE) (10) and gas chromatography–mass spectrometry (GC–MS) (11, 12).

To date, no method has been reported for the determination of atenolol by HPLC in humans in Turkey. Therefore, the objective of this work was to determine atenolol levels in patients with hypertension. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to International Conference on Harmonization (ICH) guidelines (13).

The advantages of the present method include a short run time and a simple and single-step extraction procedure using inexpensive chemicals. Also, this method was used to assay atenolol in plasma samples obtained from patients with hypertension. Additionally, this method was efficient in analyzing large amounts of plasma obtained for pharmacokinetic study after therapeutic doses of atenolol.

Experimental

Chemicals and reagents

Atenolol and tensinor tablets (50 mg atenolol) were donated from Abdi Ibrahim Pharmaceutical Industry (Istanbul, Turkey). Metoprolol tartrate, ethylacetate, hexane, dichloromethane, acetonitrile, butanol and chloroform were purchased from Sigma–Aldrich (St. Louis, MO). HPLC-grade organic solvents were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade. Distilled water was prepared as required by using an aquaMAX ultra, Young Instrument (Korea) ultrawater purification system. Human plasma was obtained from Yakutiye Blood Bank (Erzurum, Turkey).

Apparatus and analytical conditions

A Perkin Elmer series 200 HPLC system equipped with programmable fluorescence detector and Total Chrom Chromatography Data System software was used (Perkin Elmer Life and Science, Shelton, CT). The HPLC mobile phase was composed of methanol–water (50:50, v/v) containing 0.1% trifluoroacetic acid (TFA). Separation was achieved using an Ace C18 reverse-phase column (5 μm, 4.6×250 mm i.d.) with a guard column (5 μm, 4 × 3 mm i.d., Phenomenex) packed with the same material at a flow rate of 1 mL/min. The eluent was monitored by fluorescence detection at 276 nm (excitation) and 296 nm (emission).

Solutions

A stock solution (1 mg/mL) of atenolol was prepared in methanol. The initial stock solution was further diluted in methanol to produce solutions of atenolol (1 μg/mL). Calibration standards of atenolol at concentrations of 5, 10, 25, 50, 75, 100 and 150 ng/mL were prepared by spiking appropriate amounts of the standard solutions in blank plasma. Standard solutions were stored at +4°C. The internal standard (IS) stock solution was made at an initial concentration of 1 mg/mL. The IS working solution (10 μg/mL) was made from the stock solution using methanol dilution.
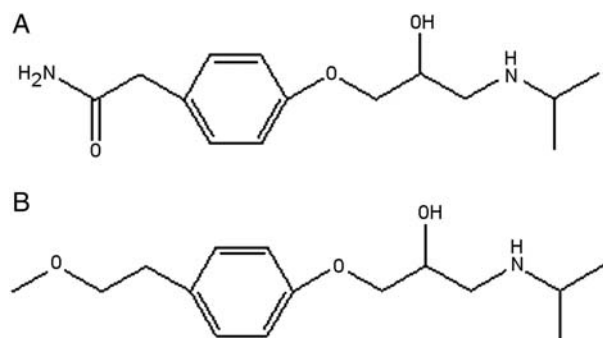
Figure 1. Chemical structures of atenolol (A) and metoprolol, IS (B).

Preparation of quality control samples

The concentrations of atenolol were 12.5, 60.0 and 125.0 ng/mL in human plasma to represent low, middle and high quality controls, respectively. Appropriate volumes from the stock solution of atenolol were added to normal human plasma to get low, middle and high quality control samples, respectively, and stored at $-20°C$. The quality control samples were taken from storage for analysis to determine intra-day and inter-day precision and accuracy.

Extraction procedure

A small amount of atenolol is bound (6–16%) to proteins to plasma (2). Therefore, several solvents (ethylacetate, hexane, dichloromethane, acetonitrile, butanol and chloroform) were tested for the extraction. Finally, a chloroform and butanol mixture (4:1, v/v) proved to be the most efficient in extracting atenolol from human plasma. A 0.5-mL aliquot plasma sample was transferred to a 10-mL glass tube. A suitable amount of standard atenolol solutions were added, together with the IS solution (20 μL, 10 μg/mL) and 0.5 mL 1M sodium hydroxide solution. After vortex mixing for 5 s, 5 mL of chloroform and butanol was added (4:1, v/v); the mixture was vortexed for 30 s and then centrifuged at $3,000 \times g$ for 7 min. The organic layer was transferred into another another 5-mL tube and evaporated to dryness under stream of nitrogen gas at $40°C$. The residue was reconstituted in 1 mL methanol, and a 20-μL aliquot was injected into the HPLC system.

Collection of samples

Before the study, the clinical protocol was approved by the Ethics Committee of Faculty of Medicine, Ataturk University (2009/Number 41). All volunteer patients were given written informed consent to participate in the study according to the principles of the Declaration of Helsinki. The patients who submitted the agreements to attend this project were medically examined and six patients with hypertension were selected (36.3 ± 1.25 years; 74.3 ± 1.84 kg; 174 ± 4.92 cm) for the pharmacokinetics study for atenolol. The subjects were required to abstain from taking any other drug for seven days prior the start of the test. They were also forbidden to smoke or drink alcohol or xanthine-containing beverages from 24 h before the beginning of the study until its end. Six patients with hypertension received an oral tablet (tensinor 50 mg) containing 50 mg of atenolol. They were then allowed to drink water. The total amount of water drunk during the day was 1,500 mL. The volunteers were sitting during lunch and had normal activity (standing or sitting) during the study, but were never in a supine position during the 12 h after administration. Blood samples were taken into EDTA tubes at 0, 0.5, 1, 2, 3, 4, 6, 8 and 12 h after oral administration. The blood was centrifuged $3,000 \times g$ for 10 min at ambient temperature and extracted.

Assay Validation

Linearity

Calibration curves were prepared by adding a known amount of atenolol (5, 10, 25, 50, 75, 100 and 150 ng/mL) to 0.5 mL of blank plasma. An aliquot of 20 μL of the IS solution (200 ng/mL) was added to each sample. The samples were extracted as described previously. The standard curves were constructed by plotting the peak area ratio of atenolol and IS on the Y-axis and concentration of atenolol on the X-axis. Linearity was assessed by a weighted (1/C) least-squares regression analysis.

Precision and accuracy

For the calculation of the intra-day precision and accuracy, six replicates of quality control samples (12.5, 60.0 and 125.0 ng/mL) were extracted as described previously and the concentrations were calculated from the standard curve. For the calculations of inter-day precision and accuracy, six replicates of quality control samples were analyzed on three consecutive days along with the standard calibration curve.

Sensitivity

The sensitivity was evaluated by the limit of quantification (LOQ), the lowest concentration of the plasma spiked with atenolol in the calibration curve. The limit of detection (LOD) was determined as the lowest concentration, which gives a signal-to-noise ratio of 3 for atenolol.

Specificity

Preparation of plasma samples was processed by the liquid–liquid extraction procedure. The samples were chromatographed to determine to which extent endogenous plasma components may contribute to the peak interference at retention times of atenolol and IS. Commonly prescribed drugs were analyzed for possible interference. The retention times were determined for these drugs under the chromatographic conditions for the atenolol assay.

Recovery

To calculate recovery of the extraction procedure, six replicates of quality control samples of atenolol (12.5, 60.0 and 125.0 ng/mL) were extracted and analyzed. The peak area was compared with the same concentrations of unextracted standards of atenolol reconstituted in methanol. The average value

of the area was taken into consideration to calculate the recovery. The recovery of IS was also calculated in a similar way.

Stability

In bench-top stability, three replicates of low and high controls of atenolol (10 and 150 ng/mL) were analyzed at 0 and 6 h at room temperature and the deviation was calculated. In autosampler stability, three replicates of high and low quality control samples were analyzed at 0, 12 and 24 h by keeping in the autosampler at 10°C and the deviation was calculated.

In freeze-thaw stability, three replicates of low and high quality control samples of atenolol were prepared, frozen at −20°C and analyzed after one, two and three freeze-thaw cycles. In dry extract stability, three replicates of high and low quality control samples were prepared. After evaporating the organic phase, the tubes were stored at −20°C and analyzed after 24 h by reconstituting with 1 mL of methanol and injecting 20 μL into the HPLC system. The mean concentration of 24 h samples was compared with that of the sample analyzed at 0 h. Long-term stability was examined for 14 days by taking three replicates of high and low quality control samples. The mean concentration was taken into consideration, which was compared with zero-day sample concentration.

Data analysis

The present method was used to determine the plasma concentration of atenolol. The peak plasma level (C_{max}) is the highest observed concentration and T_{max} is the corresponding time of this concentration. The areas under the plasma concentration-time curves (AUC) were calculated with the linear trapezoidal rule. The $AUC_{0-\infty}$ was calculated by dividing the last measured concentration (C_t) by elimation rate constant (k_{el}) and adding the result to the AUC_{0-t}. The elimination rate constant was calculated by the least-squares regression using the last five time points of each curve. The apparent elimination half-life was the quotient of the natural logarithm of 2 and the elimination rate constant (15).

Results

Validation of the method

To evaluate the validation of the present method, parameters such as specificity, linearity, precision, accuracy, LOD and LOQ, recovery and stability were investigated according to ICH validation guidelines (13).

Specificity

Plasma samples obtained from patients with hypertension were assessed by the procedure, as described previously, and compared with respective plasma samples to evaluate the specificity of the method. Drug-free plasma was spiked with therapeutic concentrations of such drugs, which included various drugs (carvedilol, nebivolol, ibuprofen, naproxen, mexiletine, rofecoxib, medazepam, diazepam, disulfiram, estradiol valerate and medroxyprogesterone acetate). The retention times for these drugs under the chromatographic conditions for the

Table I

Results of Regression Analysis of the Linearity Data of Atenolol in Human Plasma

	Mean ± SD ($n = 3$) Plasma
Slope	0.1964 ± 0.0216
Intercept	0.0221 ± 0.0017
Correlation coefficient (r)	0.9981 ± 0.0004

Table II

Intra-Day and Inter-Day Precision and Accuracy of Atenolol in Human Plasma ($n = 6$)

Sample	Concentration (ng/mL) Added	Found (mean ± SD)	%RSD	%REM
Plasma	12.5	12.16 ± 0.361	2.97	−2.72
Intra-day	60.0	61.31 ± 1.618	2.64	2.18
	125.0	118.12 ± 2.292	1.94	−5.50
Inter-day	12.5	12.25 ± 0.747	6.09	−2.00
	60.0	58.46 ± 2.847	4.87	−2.57
	125.0	118.87 ± 4.190	3.52	−4.90

atenolol assay were determined and found not to interfere with atenolol and IS retention time.

System suitability

A system suitability test of the HPLC system was performed before each validation run. Five replicate injections of a system suitability/calibration standard and one injection of a control standard were made. Area percent relative standard deviation (%RSD), tailing factor and efficiency for the five suitability injections were determined. For all sample analyses, the tailing factor was ≤1.09 and efficiency ≥2,165. The %RSD of peak area and retention time (t_R) for atenolol are within 1.92%, indicating the suitability of the system.

Linearity

The calibration curves were obtained by plotting the peak area ratio of atenolol to IS against the respective concentrations. Three calibration curves were used to show linearity over the range of 5–150 ng/mL (Table I). The correlation coefficient was found to be above 0.99.

Precision and accuracy

The method indicated very good precision and accuracy. Data on the intra-day and inter-day precision and accuracy for atenolol from plasma samples are shown in Table II. The intra-day and inter-day precisions were measured to be within 2.97 and 6.09% for plasma, respectively.

LOD and LOQ

The LOD and LOQ were determined by injecting progressively low concentrations of the standard solution under the chromatographic conditions. The LOD was defined as a signal-to-noise (S/N) ratio of 3:1 and the LOD was defined as an S/N ratio of 10. The LOD and LOQ values for atenolol were found to be 1.5 and 5 ng/mL, respectively.

Table III

Recovery of Atenolol in Human Plasma ($n = 6$)

	Concentration (ng/mL)		%Recovery	%RSD
Sample	Added	Found (mean ± SD)		
Plasma	12.5	12.21 ± 0.358	97.7	2.93
Intra-day	60.0	61.18 ± 1.542	101.9	2.52
	125.0	119.52 ± 2.346	95.6	1.96
	12.5	12.32 ± 0.749	98.6	6.07
Inter-day	60.0	58.46 ± 2.427	97.4	4.15
	125.0	119.92 ± 4.094	95.9	3.41

Table IV

Stability of Atenolol in Human Plasma ($n = 3$)

|	Recovery (mean ± SD)	
	Plasma concentration (ng/mL)	
Treatment	10	150
Bench top stability for 6 h	92.16 ± 3.244	88.26 ± 5.263
Autosampler stability for 24 h	93.16 ± 4.642	90.26 ± 3.871
Three freeze-thaw cycles	91.15 ± 4.342	92.25 ± 3.726
Dry extract stability for 24 h	101.16 ± 3.462	98.06 ± 5.462
Stored at RT for 24 h*	89.64 ± 3.623	90.16 ± 6.322
Stored at −20°C for 24 h	87.65 ± 2.436	89.26 ± 4.087
Stored at −20°C for 2 weeks	88.06 ± 6.436	89.34 ± 5.618

*RT: room temperature.

Recovery

The mean extraction recovery of atenolol from human plasma was 98.4%. The mean relative recovery for IS at 200 ng/mL was 93.1 ($n = 6$). Recovery data are shown in Table III. Atenolol was extracted from plasma with a solid-phase extraction procedure described by Iha *et al.* (5). This method is also the most comprehensive method that can extract atenolol in a single extraction procedure.

Stability

The percentage variation observed in bench-top stability, autosampler stability, three freeze-thaw cycles and dry extract stability were within the limit of ± 15% (Table IV). The results of bench-top stability suggested that atenolol was stable at room temperature for 6 h. Autosampler stability showed that extracted samples were stable for 24 h at 10°C. The results of freeze-thaw and dry extract stability showed that atenolol was stable up to three freeze-thaw cycles and up to 24 h at −20°C in the form of dry extract, respectively.

Discussion

Method development was focused on the optimization of column detection, sample preparation and chromatographic separation. A reversed-phase column (C18) can be used for the separation of non-ionic and ion forming non-polar to medium polar substances, while normal-phase chromatography can be used for the separation of non-ionic and/or non-polar substances. The majority of ionizable pharmaceutical compounds can be sufficiently separated on a C18 column (15, 16). Thus,
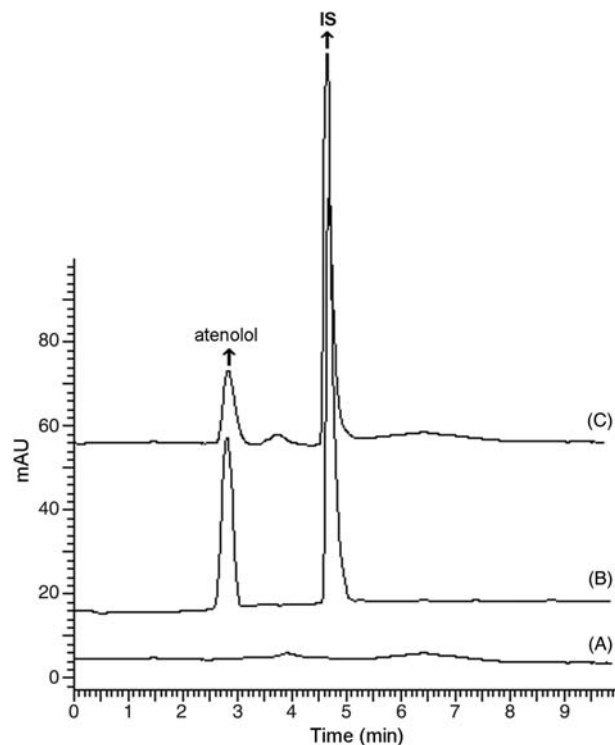

Figure 2. Representative chromatograms of (a) drug-free plasma, (b) the plasma spiked with atenolol (100 ng/mL) and IS (200 ng/mL), (c) the plasma obtained at 6 h after a single oral dose of 50 mg atenolol.

atenolol can be satisfactorily separated by reversed-phase chromatography.

Several tests were performed for optimizing the components of mobile phase to achieve good chromatographic peak shape and resolution. The test results showed that the solvent system of water could improve the peak shapes of atenolol. Good separation of target compounds and a short run time were obtained using a mobile phase system of methanol–water (50:50, v/v) containing 0.1% TFA. The retention time of atenolol (2.75 min) was much shorter than that studied by Giachetti *et al.* (4) and Iha *et al.* (5). However, the mobile phase in the proposed method, methanol–water instead of buffered systems, has been used in previously reported HPLC methods (2, 3, 4). Liquid–liquid extraction was used for the sample preparation in this work. Several solvents (ethylacetate, dichloromethane, acetonitrile, butanol and chloroform) were tested, and a chloroform–butanol mixture (4:1, v/v) proved to be the most efficient in extracting atenolol from human plasma. After the extraction procedure, the dry residue was dissolved in 1 mL methanol. A 20 μL sample was injected into the HPLC system.

Metoprolol was chosen as IS because it showed similar chromatographic behavior to atenolol with no interference by admixture in human plasma. Satisfactory peak resolution and reasonable retention of the drug and IS were obtained using the reverse-phase C18 column. Representative chromatograms of plasma spiked with atenolol (100 ng/mL) and IS (200 ng/mL) (Figure 2A) and the plasma obtained at 6 h after a single dose of 50 mg (Figure 2B) atenolol are given in Figure 2. No
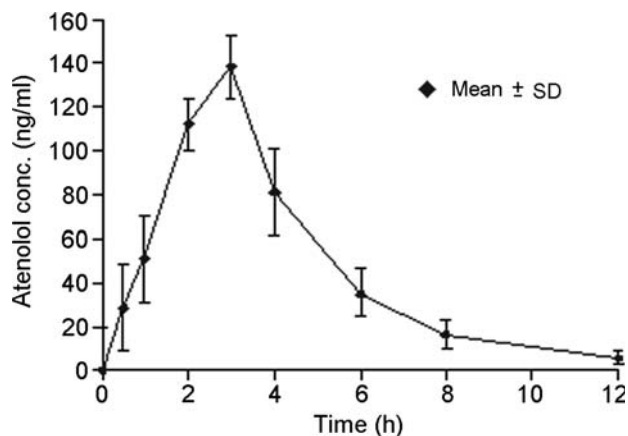
Figure 3. Mean plasma atenolol concentration-time profile for six patients after a single oral dose of atenolol, 50 mg.

interference in the chromatogram of drug-free plasma was observed.

When this method is applied to plasma samples, its sensitivity was found to be adequate for pharmacokinetic studies. The present method has several advantages over the reported methods. The method is as good as or superior to those reported in the other papers (4–6).

The calibration curve of atenolol was linear over the concentration range of 5–150 ng/mL for plasma, which is as good as or superior to that reported in other papers (3, 5, 6, 8, 10).

Atenolol was extracted from plasma with a solid-phase extraction procedure described by Iha *et al.* (5). This method is also the most comprehensive method that can extract atenolol in a single extraction procedure. The mean recovery is better for plasma than those of the studies reported by Chatterjee *et al.* (3), Miller *et al.* (6) and Arias *et al.* (10).

Pires de Abreu *et al.* (2) reported an HPLC method with fluorescence detection for the analysis of atenolol in human plasma. The calibration curve of HPLC method was linear for atenolol in the range 25–800 ng/mL. Intra-day and inter-day precision, expressed as the RSD, were less than 8.9%, and accuracy (relative error) was beter than 10.1%. The LOQ and LOD of the method were 25 and 10 ng/mL, respectively. The LOQ and LOD of the proposed HPLC method are lower than those in earlier reported works (2, 4, 6). However, the mobile phase in the proposed method was methanol–water (50:50, v/v) containing 0.1% TFA, instead of the buffered systems that were used in previously reported HPLC methods (2, 3, 4). Therefore, flushing of the column after analysis is not required. Also, 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 mean AUC, C_{max} and T_{max} of atenolol obtained from patients with hypertension (587.32 $\pm$ 95.028 h ng/mL, 138.7 $\pm$ 13.430 ng/mL and 3.0 $\pm$ 0.659 h, respectively) were lower than the reported values by Pires de Abreu *et al.* under a comparable study design (2), at least in part due to differences in ethnic groups, pharmaceutical forms and analytical method.

Li *et al.* (9) reported an LC method with tandem mass detection for the analysis of atenolol in human plasma. The

calibration curve of LC–MS-MS method was linear for atenolol in the range 10–2,000 ng/mL. Intra-day and inter-day precision, expressed as the RSD, were less than 5.3%, and accuracy (relative error) was beter than 8.0%. Detection using LC–MS-MS is a more sensitive approach, but it is costly and not yet available for every laboratory.

The mean plasma concentration-time profiles of atenolol following administration of single oral dose of a 50-mg tablet to six patient male subjects are shown in Figure 3, and a summary of the pharmacokinetic parameters is presented in Table V. Pharmacokinetic parameters obtained using the proposed method are in agreement with those of the previously reported studies (4, 5, 11) Furthermore, a low volume of plasma (0.5 mL) is used in the proposed method, which can be advantageous in clinical pharmacokinetic studies. The other methods used 1.0 mL of plasma (4, 5).

Conclusion

In the present work, a new, simple and sensitive HPLC method was developed. Also, the HPLC method was completely validated by using selectivity, stability, sensitivity, linearity, accuracy and precision parameters for the determination of atenolol in human plasma. The method was found to be linear over an analytical range of 5–150 ng/mL. Additional advantages of this method include a small sample volume (0.5 mL), good extraction recovery from plasma and a readily available internal standard. Therefore, the proposed method can be used as a therapeutic drug monitoring method in clinics to check the plasma concentration of atenolol in patients with hypertension.

Acknowledgment

References

1. Al-Ghannam, S.M.; A simple spectrophotometric method for the determination of β-blockers in dosage forms; *Journal of Pharmaceutical and Biomedical Analysis*, (2006); 40: 151–156.
2. 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 Journal*, (2003); 5(2): 116–122.
3. Chatterjee, D.J., Li, W.Y., Hurst, A.K., Koda, R.T.; High-performance liquid chromatographic method for determination of atenolol from human plasma and urine: simultaneous fluorescence and ultraviolet detection; *Journal of Liquid Chromatography*, (1995); 18: 791–806.

4. Giachetti, C., Tencoti, A., Canali, S., Zanolo, G.; Simultaneous determination of atenolol and chlorthalidone in plasma by high-performance liquid chromatography Application to pharmacokinetic studies in man; *Journal of Chromatography B*, (1997); 698: 187–194.

5. Iha, M.H., Martinez, A.S., Bonato, P.S.; Enantioselective analysis of atenolol in biologic fluids: comparison of liquid-liquid and solid-phase extraction methods; *Journal of Chromatography B*, (2002); 767: 1–9.

6. Miller, R.B.; A validated high-performance liquid chromatographic method for the determination of atenolol in whole blood; *Journal of Pharmaceutical and Biomedical Analysis*, (1991); 9: 849–853.

7. Morris, R.G., Saccoia, N.C., Sallustio, B.C., Zacest, R.; Improved high-performance liquid chromatography assay for atenolol in plasma and urine using fluorescence detection; *Therapeutic Drug Monitoring*, (1991); 13: 345–349.

8. Chiu, F.C., Zhang, J.N., Li, R.C., Raymond, K.; Efficient assay for the determination of atenolol in human plasma and urine by high-performance liquid chromatography with fluorescence detection; *Journal of Chromatography B*, (1997); 691: 473–477.

9. Li, W., Li, Y., Francisco, D.T., Naidong, W.; Hydrophilic interaction liquid chromatographic tandem mass spectrometric determination of atenolol in human plasma; *Biomedical Chromatography*, (2005); 19: 385–393.

10. Arias, R., Jimenez, R.M., Alonso, R.M., Telez, M., Arrieta, I., Flores, P., etc.; Determination of the β-blocker atenolol in plasma by capillary zone electrophoresis; *Journal of Chromatography A*, (2001); 916: 297–304.

11. Yilmaz, B., Arslan, S.; GC-MS determination of atenolol plasma concentration after derivatization with N-methyl-N-(trimethylsilyl)-trifluoroacetamide; *Chromatographia*, (2009); 70: 1399–1404.

12. Amendola, L., Molaioni, F., Botre, F.; Detection of beta-blockers in human urine by GC-MS-MS-EI: perspectives for the antidoping control; *Journal of Pharmaceutical and Biomedical Analysis*, (2000); 23: 211–221.

13. International Conference on Harmonization; Validation of analytical procedures. In Proceedings of the ICH. *Commission of the European Communities*, (1996).

14. Yilmaz, B., Erol, Z.; Determination of carvedilol in rabbit plasma by gas chromatography-mass spectrometry and its application to pharmacokinetic study; *Asian Journal of Chemistry*, (2011); 23: 49–53.

15. Yilmaz, B., Asci, A., Palabiyik, S.S.; HPLC method for determination of diclofenac in human plasma and its application to a pharmacokinetic study in Turkey; *Journal of Chromatographic Science*, (2011); 49: 422–427.

16. Yilmaz, B., Asci, A., Arslan, S.; Determination of metoprolol in human plasma and urine by high-performance liquid chromatography with fluorescence detection; *Journal of Separation Science*, (2010); 33: 1904–1908.