Journal of Chromatographic Science 2013;51:128–132
doi:10.1093/chromsci/bms116 Advance Access publication June 22, 2012

Article

Determination of Atenolol in Human Plasma by HPLC with Fluorescence Detection: Validation and Application in a Pharmacokinetic Study

Marios Spanakis and Ioannis Niopas*

Department of Pharmacognosy and Pharmacology, School of Pharmacy, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece

*Author to whom correspondence should be addressed. Email: niopas@pharm.auth.gr

Received 28 April 2010; revised 21 November 2010

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 101.6%, 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.

Introduction

Atenolol is a hydrophilic cardioselective β1-adrenergic receptor blocking agent without membrane stabilizing or intrinsic sympathomimetic activities, widely used for the treatment of hypertension, angina pectoris and cardiac arrhythmias (1–3). The cardioselectivity of atenolol is not absolute and at higher doses inhibits β2-adrenergic receptors, resulting in bronchospasm (4). Atenolol is absorbed quickly but not completely by the gastrointestinal tract. After oral administration of atenolol, approximately 50–60% of the dose is absorbed and the rest is excreted unchanged in the feces. Atenolol undergoes little or no metabolism and is eliminated primarily by renal excretion (2).

The conduct of pharmacokinetic studies in humans after oral administration of atenolol requires a specific and sensitive analytical method for the quantitative determination in plasma samples. In attempting to establish a high-performance liquid chromatography (HPLC) method in this laboratory, we experienced difficulties in reproducing other methods, particularly in attaining sufficient selectivity and sensitivity suitable for pharmacokinetic studies.

Various analytical methods have been reported for the determination of atenolol either alone or in combination with other drugs, especially β-blockers or diuretics, in biological matrices, such as HPLC combined with fluorescence detection (5–15) or ultraviolet (UV) absorption (16–18), as well as liquid chromatography–tandem mass spectrometry (LC-MS/MS) (19–21). HPLC with fluorescence detection and LC-MS/MS are the preferred methods in terms of sensitivity and selectivity.

Some of the reported analytical methods did not focus exclusively on the determination of atenolol, but were used for the simultaneous determination of other drugs such as β-blockers (12, 14, 16, 17, 20), diuretics (7, 20) or antiarrhythmics (21). Some required relatively large plasma samples (1.0 to 2.0 mL) (6–8, 10), some were less sensitive than the current method (6, 9, 11, 15, 18), some required complex and expensive instrumentation (19–21) and some were poorly reproducible, especially in lower concentration ranges, and were not directly applicable to pharmacokinetic studies (11, 13).

Therefore, the aim of the current study was to develop and validate a simple, selective, sensitive, accurate and precise HPLC method with fluorescence detection using 0.5-mL plasma samples for the determination of atenolol in human plasma that is capable of conducting pharmacokinetic or bioequivalence studies after oral administration of atenolol.

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 an ISCO 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 cyano analytical column (100 × 4.6 mm i.d.,

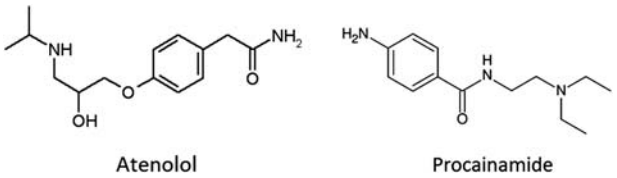

Figure 1. Chemical structures of atenolol and procainamide (internal standard).

5 μm particle size) (Perkin-Elmer). The mobile phase consisted of a mixture of an aqueous solution containing 5 mM triethylamine, 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 chromatographic peaks were measured by fluorescence detection at 229 and 309 nm for l_{ex} and l_{em}, respectively. Quantitation of atenolol was determined by peak height ratio comparisons with standard curves, which were prepared daily using drug-free plasma samples.

Standard solutions

Stock solutions of atenolol and the internal standard, procainamide, were prepared daily by dissolving appropriate amounts of the compounds in milli-Q water to achieve concentrations of 20 and 300 μg/mL, respectively. The stock solution of atenolol was further diluted with milli-Q water to prepare working solutions containing 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10 μg/mL of atenolol. The stock solution of the internal standard was also diluted with milli-Q water to give the internal standard working solution of 25 μg/mL. Calibration standard samples were freshly prepared in 0.5 mL of human plasma by spiking with 50 μL of the atenolol working solutions and 25 μL of the internal standard working solution to yield concentrations corresponding to 10, 25, 50, 100, 250, 500 and 1,000 ng/mL of atenolol and 1.25 μg/mL of internal standard.

Quality control samples

Volumes of 25 mL of human plasma were spiked with appropriate 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 approximately 1 mL into one-dram vials capped tightly and placed at −20°C, pending analysis. These samples were used in the analysis of plasma samples as QCs for the purpose of checking precision, 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 internal 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 centrifuged 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 conjunction with the linearity studies by assaying on three separate occasions using three QC samples at each of the three concentrations (50, 100 and 500 ng/mL). Concentrations of atenolol in QC samples were determined by application of the appropriate 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 deviation (RSD) was used to express assay precision. The lower limit of quantification (LLOQ) was defined as the lowest quantifiable concentration on the calibration curve at which both accuracy 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 replicate 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 determined 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 considered 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

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 (C_{max}) value and the corresponding time that the latter is marked (T_{max}) 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 ($t_{1/2}$) 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 (AUC_{0-t}) was estimated by the use of the linear trapezoidal method, according to the following equation:

$$AUC_{0-t} = \frac{1}{2}\sum_{i=0}^{n-1}(t_{i+1} - t_i)(C_i + C_{i+1})$$

where t_i is the ith time point, C_i is the ith available concentration and n is the number of data points. The area under the plasma concentration−time curve extrapolated to infinitive time ($AUC_{0-\infty}$) was estimated by the following equation:

$$AUC_{o-\infty} = AUC_{0-t} + \frac{C_n}{k}$$

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.

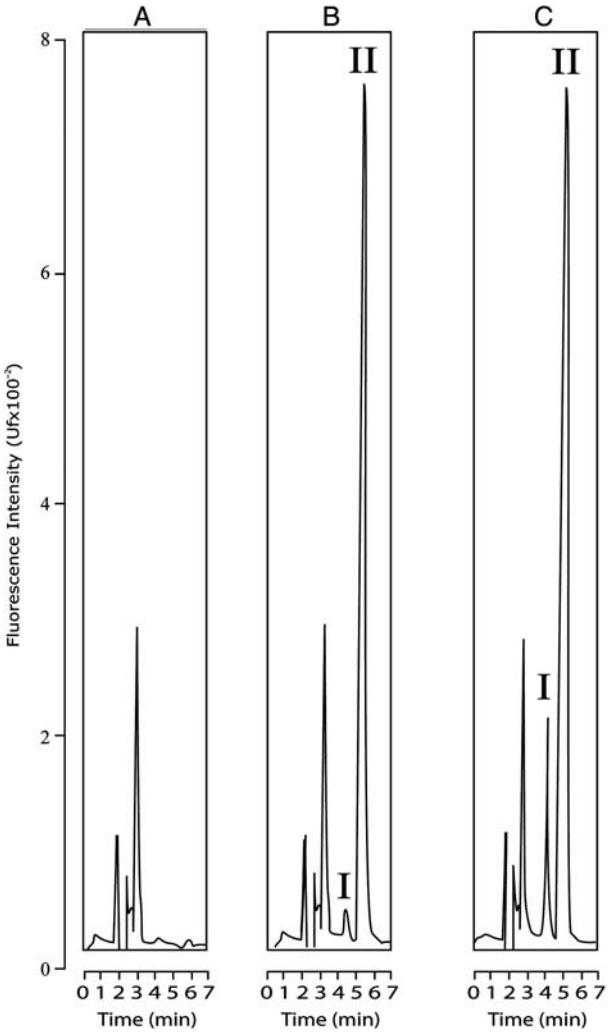

Figure 2. Typical chromatograms for: blank plasma (A); standard solution at LLOQ (10 ng/mL) (B); plasma sample obtained from a volunteer 16 h after a single oral dose of 100 mg of atenolol, containing 98.7 ng/mL atenolol and 1.25 μg/mL internal standard (C). Peaks: I, atenolol; II, internal standard (procainamide).

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

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

Nominal concentration (ng/mL)	Accuracy* (%)	Precision (RSD, %)
50	101.6 ± 6.5	6.4
100	101.0 ± 3.7	3.7
500	99.6 ± 3.7	3.8

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

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 procainamide, pKa 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 procainamide, 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 I). 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 curve after oral administration of a 100 mg tablet of atenolol to 18 healthy subjects is illustrated in Figure 3. Pharmacokinetic analysis of atenolol plasma concentration–time data provided the following pharmacokinetic parameters (mean ± SD): $AUC_{0-\infty}$ 5,440.1 ± 1,131.0 ng.h/mL, AUC_{0-t} 5,234.2 ± 1,147.7 ng.h/mL, C_{max} 537.1 ± 112.7 ng/mL, T_{max} 3.4 ± 1.0 h and $t_{1/2}$ 6.7 ± 1.2 h. The observed values of the pharmacokinetic parameters were comparable to those reported for atenolol in previous studies (24).

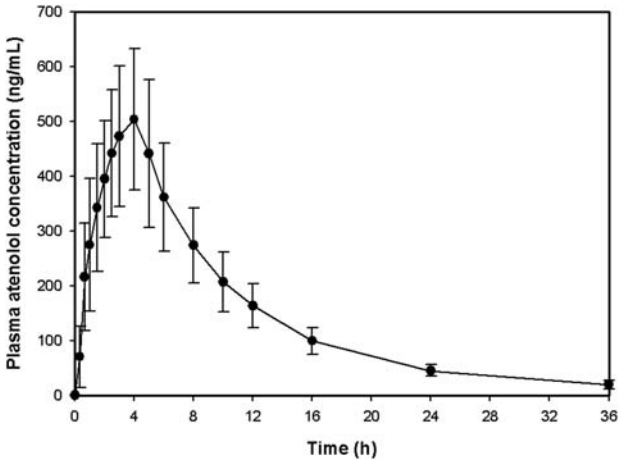

Figure 3. Mean ± SD of plasma atenolol concentration–time curve following a single oral dose of 100 mg atenolol to 18 healthy subjects.

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

1. Wadsworth, A.N., Murdoch, D., Brogden, R.N.; Atenolol: A reappraisal of its pharmacological properties and therapeutic use in cardiovascular disorders; *Drugs*, (1991); 42: 468–510.
2. Physicians' desk reference, 52nd ed. Medical Economics Data, Montvale, NJ, (1999), p. 3183.
3. Taeschner, W., Vozeh, S.; Avery's drug treatment, 4th ed. Speight, T.M., Holford, N.H.G., (eds). Adis International, Auckland, New Zealand, (1997), p. 1639.
4. Fitzgerald, J.D., Ruffin, R., Smedstad, K.G., Roberts, R., McAinsh, J.; Studies on the pharmacokinetics and pharmacodynamics of atenolol in man; *European Journal of Clinical Pharmacology*, (1978); 13: 81–89.
5. Leite, F.S., Pereira, V.A., Omosako, C.E., Carmona, M.J., Auler, J.O., Santos, S.R.; A micromethod for the quantification of atenolol in plasma using high-performance liquid chromatography with fluorescence detection: Therapeutic drug monitoring of two patients with severe coronary insufficiency before cardiac surgery; *Therapeutic Drug Monitoring*, (2006); 28: 237–244.

6. Braza, A.J., Modamio, P., Marino, E.L.; Two reproducible and sensitive liquid chromatography methods to quantify atenolol and propranolol in human plasma and determination of their associated analytical error functions; *Journal of Chromatography B*, (2000); 738: 225–231.
7. Giachetti, C., Tenconi, 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.
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. 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 and Related Technologies*, (1995); 18: 791–806.
10. 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.
11. 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.
12. Buhring, K.U., Garbe, A.; Determination of the new beta-blocker bisoprolol and of metoprolol, atenolol and propranolol in plasma and urine by high-performance liquid chromatography; *Journal of Chromatography*, (1986); 382: 215–224.
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.
14. Harrison, P.M., Tonkin, A.M., McLean, A.J.; Simple and rapid analysis of atenolol and metoprolol in plasma using solid-phase extraction and high-performance liquid chromatography; *Journal of Chromatography*, (1985); 339: 429–433.
15. Weddle, O.H., Amick, E.N., Mason, W.D.; Rapid determination of atenolol in human plasma and urine by high-pressure liquid chromatography; *Journal of Pharmacy Science*, (1978); 67: 1033–1035.
16. Delamoye, M., Duverneuil, C., Paraire, F., de Mazancourt, P., Alvarez, J.C.; Simultaneous determination of thirteen beta-blockers and one metabolite by gradient high performance liquid chromatography with photodiodo-array UV detection; *Forensic Science International*, (2004); 141: 23–31.
17. Mislanova, C., Hutta, M.; Influence of various biological matrices (plasma, blood microdialysate) on chromatographic performance in the determination of beta-blockers using an alkyl-diol silica pre-column for sample clean-up; *Journal of Chromatography B*, (2001); 765: 167–177.
18. Verghese, C., McLeod, A., Shand, D.; Rapid high-performance liquid chromatographic method for the measurement of atenolol in plasma using UV detection; *Journal of Chromatography*, (1983); 275: 367–375.
19. 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.
20. Gonzalez, O., Iriarte, G., Rico, E., Ferreiros, N., Maguregui, M.I., Alonso, R.M., *et al.*; LC–MS/MS method for the determination of several drugs used in combined cardiovascular therapy in human plasma; *Journal of Chromatography B*, (2010); 878: 2685–2692.
21. Li, S., Liu, G., Jia, J., Liu, Y., Pan, C., Yu, C., *et al.*; Simultaneous determination of ten antiarrhythmic drugs and a metabolite in human plasma by liquid chromatography-tandem mass spectrometry; *Journal of Chromatography B*, (2007); 847: 174–181.
22. U.S. Department of Health and Human Services Food and Drug Administration (FDA). Center for Drug Evaluation and Research (CDER). Center for Veterinary Medicine (CVM). Guidance for Industry: Bioanalytical Method Validation, (2001).
23. Rowland, M., Tozer, T.N.; Clinical pharmacokinetics: Concepts and applications, 3rd ed. Lea & Febiger, Baltimore, MD, (1995).
24. Najib, N.M., Idkaidek, N., Adel, A., Mohammed, B., Al-Masri, S., Admour, I., *et al.*; Comparative bioavailability of two brands of atenolol 100mg tablets (tensotin and tenormin) in healthy human volunteers; *Biopharmaceutics and Drug Disposal*, (2005); 26: 1–5.