Determination of Atenolol in Human Urine by Gas Chromatography–Mass Spectrometry Method

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Abstract

A sensitive and efficient method was developed for the determination of atenolol in human urine by gas chromatography–mass spectrometry (GC–MS). Atenolol and metoprolol (internal standard, IS) were extracted from human urine with a mixture of chloroform and butanol at basic pH with liquid–liquid extraction. The extracts were derivatized with N-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and analyzed by GC–MS using a capillary column. The standard curve was linear *(r = 0.99)* over the concentration range of 50–750 ng/mL. Intra- and inter-day precision, expressed as the relative standard deviation were less than 5.0%, and accuracy (relative error) was beter than 7.0%. The analytical recovery of atenolol from human urine has averaged 91%. The limit of quantification was 50 ng/mL. Also, the method was successfully applied to a patient with hypertension who had been given an oral tablet of 50 mg atenolol.

Introduction

Atenolol, a synthetic, beta1-selective (cardioselective) adrenoreceptor blocking agent, may be used alone or concomitantly with other antihypertensive agents including thiazide-type diuretics, hydralazine, prazosin, and α-methyldopa (1,2). Atenolol ($C_{14}H_{22}N_2O_3$) is available as 25, 50, and 100 mg tablets for oral administration.

To analyze atenolol in human plasma or other biological fluids, several bioanalytical methods have been reported. Atenolol was analyzed alone or simultaneously with other drugs using methods such as high-performance liquid chromatography (HPLC) (2–8), LC–tandem mass spectrometry (MS–MS) (9), capillary zone electrophoresis (10), and GC–MS (11,12).

β-blockers have similar chemical structures with highly polar functional groups that yield them unsuitable for analysis by GC methods. Recently the use of mass selective detectors with a capillary GC coupled to MS as a mode of detection has considerably increased. Suitable derivatization should improve the gas chromatographic properties of the compounds and yield compounds with mass spectra containing high relative intensity and high-mass fragments suitable for selected ion monitoring (SIM) (13).

The aim of the present study was to develop a specific, sensitive, precise, and accurate GC–MS method for analysis of atenolol in human urine. Atenolol was derivatized using silylating agent prior to GC–MS analysis. In order to increase the derivatization yield, some parameters, such as temperature, time, and solvent were tested. The proposed method was fully validated in respect to limits of detection (LOD) and quantitation (LOQ), precision, accuracy, linearity, specificity, stability, and extraction recovery parameters according to International Conference on Harmonization (ICH) guidelines (14).

Finally, the method was used to assay the atenolol in urine samples obtained from a patient with hypertension who had been given an oral tablet of Tensinor (50 mg atenolol).

Experimental

Chemicals and reagents

Atenolol standard and Tensinor tablet were kindly donated from Abdi Ibrahim Pharmaceutical Industry (Istanbul, Turkey). Metoprolol tartrate (internal standard, IS) was obtained by Sigma-Aldrich (St. Louis, MO). N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), chloroform, butanol, and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO).

Apparatus and analytical conditions

Chromatographic analysis was carried out on an Agilent 6890N gas chromatography (GC) system equipped with 5973 series mass selective detector, 7673 series autosampler, and Agilent chemstation. HP-5 MS column with 0.25-µm film thickness (30 m × 0.25 mm i.d.) was used for separation. Splitless injection was used, and the carrier gas was helium at a flow-rate of 1 mL/min. The injector volume was 1 µL. The injector and detector temperatures were 280°C. The oven temperature program was as follows: initial temperature was 150°C, held for 1 min, increased to 220°C at a rate of 20°C/min, held for 1 min, and finally to 300°C at a rate of 10°C/min and held for 1 min. The MS detector parameters were: transfer line temperature, 280°C; solvent delay, 3 min; and electron energy, 70 eV.

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Preparation of stock and standard solutions

The stock standard solutions of atenolol and IS were prepared with acetonitrile to a concentration of 5000 ng/mL and stored at –20°C under refrigeration. Standard solutions at 50, 100, 200, 300, 400, 500, and 750 ng/mL concentrations of atenolol were prepared by diluting appropriate volumes of stock solution with acetonitrile. IS working solution was prepared at final concentration of 2500 ng/mL. Preparation of urine working solutions: a suitable amount of standard atenolol solutions together with 500 ng/mL IS was spiked in 0.5 mL urine and then extracted with liquid–liquid extraction method. The quality control solutions were prepared by adding aliquots of standard working solution of atenolol to a final concentrations of 150, 375, and 600 ng/mL together with 0.2 mL of IS (500 ng/mL).

Sample preparation and derivatization procedure

Because atenolol is not stable and decomposes at high temperatures, it was derivatized before GC–MS analyses. Therefore, silylation reaction was used as derivatization method.

MSTFA is an effective trimethylsilyl donor. MSTFA reacts to replace labile hydrogens on a wide range of polar compounds with a –Si(CH3)3 (TMS) group and is used to prepare volatile and thermally stable derivatives for GC–MS (15). In this study, the purpose of the derivatization reaction is the raise of sensitivity thus the possibility of working in low concentrations has been occurred. Therefore, atenolol and IS were derivatized using MSTFA. The secondary amine (–NH) and hydroxy (–OH) groups, which render the compounds non-volatile and polar, were converted to the corrosponding silyl (–N-TMS) and (–O-TMS) groups, thereby rendering them volatile and non-polar.

To increase the derivatization yield and examine the effects of parameters such as temperature and time of derivatization reaction and solvent on derivatization reaction were investigated. Derivatization was carried out at different temperatures (room temperature, 50°C, and 75°C) and various times (5, 10, and 20 min). Yields of derivatization reactions were compared with each other. When derivatization was performed at room temperature, in 10 min maximum peak areas were quantitated.
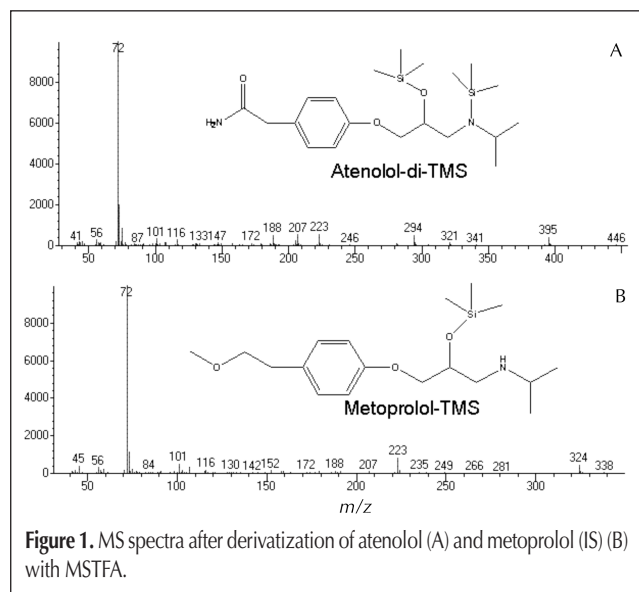
Metoprolol was selected as internal standard. It is derivatizated efficiently with an acceptable repeatability under the proposed derivatization reaction conditions for atenolol. Furthermore, any interferences from urine components were not observed.

A 0.5-mL urine sample was transferred to a 10-mL glass tube together with 0.2 mL IS solution (500 ng/mL) and 0.5 mL 1 M sodium hydroxide solution. After vortex mixing for 5 s, 3 mL of chloroform and butanol (4:1, v/v) was added, the mixture was vortexed for 30 s and then centrifuged at 3000 rpm for 7 min. The organic layer was transferred into another tube and evaporated to dryness at room temperature under nitrogen gas.

The dry residue was dissolved in 100 µL of a mixture of acetonitrile and MSTFA (50:50, v/v). The mixture was vigorously shaken and then stored at room temperature for 10 min. The mass spectra of atenolol and IS are shown in Figure 1. Then 1 µL of aliquot was injected into the GC–MS system.
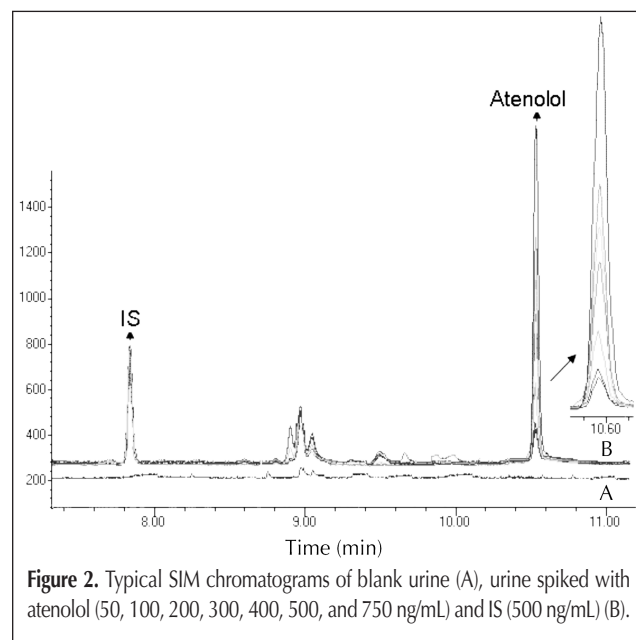
Results

Validation of the method

To evaluate the validation of the present method, parameters such as selectivity, linearity, precision, accuracy, LOD and LOQ, recovery, and stability were investigated according to ICH validation guidelines (14).

Selectivity

The selectivity of the assay was checked by comparing the chromatograms of batches of blank urine with the corresponding spiked urine. Each blank sample was tested for the observation of interference, and no endogenous interferences were encountered (Figure 2A). The fragment ion $[CH_2NHCH(CH_3)_2]^+$ (m/z 72) was used for quantitation of atenolol and IS at SIM mode. The retention time of atenolol-di-TMS and IS-TMS in human urine was approximately 10.6 and 7.8 min with good peak shape (Figure 2B).

Figure 1. MS spectra after derivatization of atenolol (A) and metoprolol (IS) (B) with MSTFA.

Figure 2. Typical SIM chromatograms of blank urine (A), urine spiked with atenolol (50, 100, 200, 300, 400, 500, and 750 ng/mL) and IS (500 ng/mL) (B).

Linearity

The calibration curves were obtained by spiking the control urine with 5000 ng/mL of the stock solution. The concentrations of the spiked atenolol in human urine were 50, 100, 200, 300, 400, 500, and 750 ng/mL with constant concentration of IS (500 ng/mL). The regression equations were calculated from the calibration graphs, along with the standard deviations of the slope and intercept on the ordinate (Table I). The correlation coefficient was found above 0.99.

Precision and accuracy

The precision of the analytical method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by analyzing spiked blank urine six times per day, at three different concentrations, which were urine quality control samples (150, 375, and 600 ng/mL). The intermediate precision was evaluated by analyzing the same urine samples once daily for three days. The relative standard deviation (RSD) of the predicted concentrations from the regression equation was taken as precision. The accuracy of this analytical method was assessed as the percentage relative error. For all the concentrations studied, intra- and inter-day relative standard deviation values were ≤ 4.9% and for all concentrations of atenolol the relative errors were ≤ 6.9%. These results were given in Table II.

LOD and LOQ

LOQ was determined as low concentration on the calibration curve with a signal-to-noise ratio of 10, a precision ≤ 20% and accuracy of 80–120% of its nominal value. LOD was also calculated as the concentration which signal-to noise ratio could be detected as 3. The LOD and LOQ values for atenolol were found to be 15 and 50 ng/mL, respectively (Table I).

Recovery

For extraction of atenolol from human urine, liquid–liquid extraction technique was tried using ethylacetate, dichloromethane, acetonitrile, butanol, and chloroform. It was observed that extraction yields were very low. The urine sample were done alkaline by 0.5 mL 1 M sodium hydroxide solution, and then atenolol was extracted from urine using 3 mL chloroform and butanol mixture (4:1, v/v).

The analytical recovery of atenolol from human urine was assessed by direct comparison of concentrations of atenolol obtained after the whole extraction and derivatization procedure by using six replicate at seven concentrations levels (50, 100, 200, 300, 400, 500, and 750 ng/mL) in the calibration graph versus standard atenolol-di-TMS solutions. The extraction recoveries of atenolol from human urine were between 87% and 95% as shown in Table III.

Matrix effect

The blank urines used in this study were from three different batches of healthy human urine. If the ratio < 85% or > 115%, a matrix effect was implied. The relative matrix effect of atenolol at three different concentrations (100, 350, and 650 ng/mL) was less than ± 8.1% (Table IV). The results showed that there was no matrix effect of the analytes observed from the matrix of urine in this study. Furthermore, a low volume of urine (0.5 mL) is used in the proposed method, which can be advantageous in clinical pharmacokinetic studies (4,5).

Stability

The stability of atenolol in human urine was studied under a variety of storage and handling conditions at low (150 ng/mL) and high (650 ng/mL) concentration levels. The short-term temperature stability was assessed by analyzing three aliquots of each of the low and high concentration samples that were

Table I. Linearity of Atenolol in Human Urine

Parameter	GC–MS
Linearity (ng/mL)	50–750
Regression equationa	$y = 0.0025x - 0.0711$
Standard deviation of slope	5.2×10^{-4}
Standard deviation of intercept	1.1×10^{-2}
Correlation coefficient	0.993
Standard deviation of correlation coefficient	5.5×10^{-3}
Limit of detection (ng/mL)	15
Limit of quantification (ng/mL)	50

* Based on six calibration curves, y: peak-area ratio, x: atenolol concentration (ng/mL).

Table II. Precision and Accuracy of Atenolol in Human Urine

Added (ng/mL)	Intra-day Found ± SD*	Intra-day Precision % RSD[†]	Intra-day Accuracy[‡]	Inter-day Found ± SD*	Inter-day Precision % RSD[†]	Inter-day Accuracy[‡]
Urine[§]						
150	148 ± 6.8	4.6	–1.3	146 ± 7.1	4.9	–2.6
375	358 ± 8.7	2.4	–4.5	349 ± 12.7	3.6	–6.9
600	588 ± 19.5	3.3	–2.0	573 ± 15.9	2.8	–4.5

* SD: Standard deviation of six replicate determinations; RSD: Relative standard deviation.
[†] Average of six replicate determinations.
[‡] Accuracy: (% relative error) (found – added)/added × 100.
[§] Urine volume (0.5 mL).

Table III. Recovery of Atenolol in Human Urine

Added (ng/mL)	Found (Mean ± SD*)	% Recovery	% [†] RSD
50	44 ± 3.2	88	7.3
100	91 ± 4.6	91	5.1
200	175 ± 7.4	88	4.2
300	283 ± 12.4	94	4.4
400	380 ± 17.3	95	4.5
500	458 ± 26.7	92	5.8
750	656 ± 35.6	87	5.4

* SD Standard deviation of six replicate determinations; RSD: Relative standard deviation
[†] Average of six replicate determinations.

thawed at room temperature and kept at this temperature for 8 h. Freeze-and-thaw stability (–20°C in urine) was checked through three cycles. Three aliquots at each of the low and high concentrations were stored at –20°C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze-and-thaw cycles were repeated three times and then analyzed on the third cycle. The long-term stability was determined by analyzing three aliquots of each of the low and high concentrations stored at –20°C for 1 week. The results of the stability studies were given in Table V, and no significant degradation of atenolol was observed under the tested conditions.

Application of the method

Prior to the study, the clinical protocol was approved by the Ethics Committee of Faculty of Medicine, Ataturk University (2009/Number 41). The method was applied to the analysis of urine samples from a study performed on a patient with hypertension treated with single oral doses of atenolol. Subject was given written informed consent to participate in the study according to the principles of the Declaration of Helsinki. The male patient with hypertension was participated in this study after provided their written informed consent. The male patient was a man who is 34 years old and 74 kg weight. He was also demanded not to smoke or drink alcohol or xanthine-containing beverages for 24 h before the beginning of the study until its end. The patient with hypertension received an oral tablet (Tensinor 50 mg) containing 50 mg of atenolol. Then, he was allowed to drink water. The total amount of water drunk during the day was 1500 mL. The patient was sitting during lunch. He had normal

activity (standing or sitting) during the study, but was never in a supine position during the 12 h after administration. Urine samples were collected at the following times: 0, 0–2, 2–4, 4–6, 6–10, and 10–12 h. Urine samples were immediately extracted and derivatized with MSTFA. Representative chromatograms obtained before and after administration of the drug are shown in Figure 3.

Discussion

Today, GC–MS is a powerful technique for highly specific and quantitative measurements of low levels of analytes in biological samples. As compared to HPLC, high-resolution capillary GC has been less frequently used. Because it requires pre-conversion of multifunctional β-blockers into thermally stable volatile derivatives. However, it has inherently high resolving power and high sensitivity with excellent precision and accuracy allowed simultaneous detection of expected and unexpected β-blockers, their metabolites and contaminants (13).

During method development, it became evident that atenolol and IS were very sensitive to matrix effects during the derivatization process in urine. Sample preparation techniques, such as liquid–liquid extraction was used in order to minimise matrix suppression effects.

GC–MS method sensitivity is not enough for the determination of atenolol in urine. For this reason, MSTFA was chosen as a chromagenic derivatization reagent. In this study, the purpose of the derivatization reaction is the raise of sensitivity thus the possibility of working in low concentrations has been occurred.

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

Calibration curve of atenolol was linear over the concentration range of 50–750 ng/mL for urine, 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 by Iha et al. (5). This method is also the most comprehensive method which can extract atenolol in a single

Table IV. Matrix Effect Evaluation of Atenolol and IS in Human Urine (*n* = 3)

Samples	Conc. level (ng/mL)	A* (Mean ± SD)	B* (Mean ± SD)	% Matrix effect
Atenolol	100	87	92	5.7
	350	323	338	4.6
	650	582	629	8.1
IS	500	447	478	6.9

* A = The amount of atenolol and IS derivatized in blank urine sample's reconstituted solution (the final solution of blank urine after extraction and reconstitution), and B = The amount of atenolol and IS derivatized with MSTFA.

Table V. Stability of Atenolol in Human Urine Under Various Storage Conditions (*n* = 3)

Storage conditions	Conc. level (ng/mL)	Calculated conc. (ng/mL)	% RSD	% Relative error
Room temperature for 8 h	150	145	5.8	–3.3
	650	633	4.4	–2.6
Three freeze-and-thaw cycles	150	143	7.4	–4.7
	650	616	6.5	–5.2
Storage conditions 1 week at –20 °C	150	141	5.8	–6.0
	650	619	6.7	–4.8
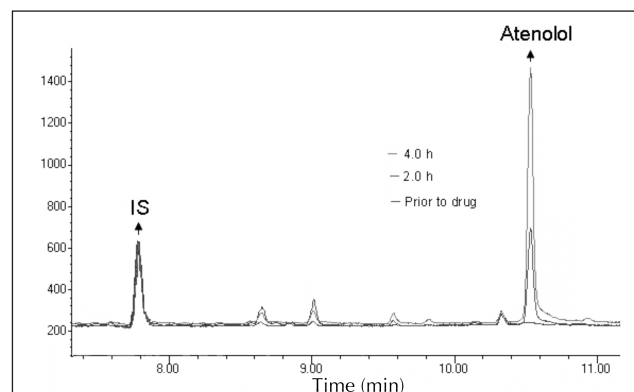
Figure 3. Typical SIM chromatograms of urine obtained from a patient at before and after oral administration of 50 mg atenolol.

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).

Li et al. (9) have reported 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 10–2000 ng/mL. Intra- and inter-day precision, expressed as the relative standard deviation (RSD) were less than 5.3%, and accuracy (relative error) was beter than 8.0%. Detection using LC–MS–MS would be a more sensitive approach but is costly and not yet available for every laboratory.

Figure 4 shows the cumulative urinary excretion curve for atenolol obtained from a patient with hipertansiyon with a single dose of Tensinor (atenolol, 50 mg). Although our results were obtained from only one subject, our results are in agreement with the literature (5).

Conclusion

A novel, simple, specific, and sensitive GC–MS method was described for analysis of atenolol in human urine. Prior to analysis, atenolol was efficiently and simply derivatized with MSTFA at room temperature for 10 min. Also, the method has good linearity, precision, accuracy, sensitivity according to the results obtained from validation data. The applicability of the method was confirmed in a patient with hypertension. In addition, the method is suitable for the pharmacokinetic study and bioavailability evaluation of atenolol and can also be used as a therapeutic drug monitoring method in clinic to check atenolol concentration in the patients with hypertension.
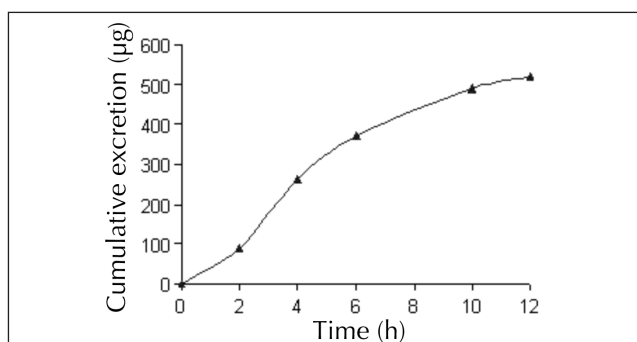
Figure 4. Cumulative urinary extraction of atenolol following a single oral dose of 50 mg atenolol.

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