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Gas Chromatography/Mass Spectrometry Method for Determination of Atenolol in Rabbit Plasma

B. YILMAZ[1,*] and V. AKBA[2]

[1]Department of Analytical Chemistry, Faculty of Pharmacy, Ataturk University, 25240 Erzurum, Turkey
[2]Criminal Police Laboratory, 25060 Erzurum, Turkey

*Corresponding author: Fax: +90 442 2360962; Tel: +90 442 2315213; E-mail: yilmazb@atauni.edu.tr

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This work describes a gas chromatography/mass spectrometry (GC/MS) method with selected-ion monitoring for determination of atenolol in rabbit plasma. Atenolol and internal standard metoprolol were extracted from plasma and cleaned up by using a single step liquid-liquid extraction. Calibration curves were linear over the concentration range 15-250 ng/mL. Intra- and inter-day precision values for atenolol in rabbit plasma were less than 7.4 and accuracy (relative error) was better than 6.4. The analytical recovery of atenolol from rabbit plasma averaged out to 90.46 %. The limit of detection (LOD) and limit of quantification (LOQ) of atenolol were 5 and 15 ng/mL, respectively. The developed and validated GC/MS method was also successfully applied to a pharmacokinetic study of atenolol in New Zealand white rabbits.

Key Words: Atenolol, Gas chromatography/mass spectrometry, Pharmacokinetics.

INTRODUCTION

β-Blockers constitute one of the most frequently prescribed groups of cardiovascular drugs. They are competitive antagonists at β-adrenergic receptor sites and 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. 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 including high performance liquid chromatography (HPLC)[2-8], LC/MS/MS[9], capillary zone electrophoresis[10] and GC/MS[11] in plasma and other biological fluids. In addition, no method is reported till date for determination of atenolol by GC/MS from rabbits which had been given atenolol. Therefore, we report a GC/MS method for determination of atenolol after a derivatization procedure in rabbit plasma using internal standard methodology.

The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to International Conference on Harmonization (ICH) guidelines[12]. The advantages of present method include simple and single step extraction procedure using inexpensive chemicals and short run time. This method was also used to a pharmacokinetic study of atenolol in rabbit plasma.

EXPERIMENTAL

Atenolol and tensinor tablet (50 mg atenolol) were kindly donated from Abdi Ibrahim Pharmaceutical Industry (Istanbul, Turkey). Metoprolol tartrate (internal standard), N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), chloroform, butanol and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Chromatographic analysis was carried out on an Agilent 6890N gas chromatography system equipped with 5973 series mass selective detector, 7673 series autosampler and Agilent chemstation (Agilent Technologies, Palo Alto, CA). HP-5 MS column with 0.25 μm film thickness (30 m × 0.25 mm i.d., USA) was used for separation. Splitless injection was used and the carrier gas was helium at a flow rate of 1 mL/min. The injector and detector temperatures were 280 ºC. The MS detector parameters were transfer line temperature 280 ºC, solvent delay 3 min and electron energy 70 eV.

Preparation of stock and working solutions: 10 mg atenolol and metoprolol tartrate (internal standard) was weighed, transferred 500 mL volumetric flask, differently. 250 mL acetonitrile was added and the flask was sonicated. The flask was filled to volume with acetonitrile (20 μg/mL). After, 5000 ng/mL standard solution was prepared by diluting with

acetonitrile appropriate volumes of 20 µg/mL stock solution and stored at -20 ºC under refrigeration. The working solutions were prepared by diluting the standard stock solution from 15-250 ng/mL. The quality control (QC) solutions were also prepared from stock solution at concentrations of 35, 125 and 225 ng/mL together with 500 ng/mL internal standard.

Sample preparation and derivatization procedure: 0.5 mL rabbit plasma was transferred to a 10 mL glass tube. After, a suitable amount of standard atenolol solutions together with 0.1 mL internal standard solution (500 ng/mL) and 0.5 mL 1 M sodium hydroxide solution were added. 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 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 N-methyl-N-trimethylsilyl-trifluoroacetamide (50:50, v/v). The mixture was vigorously shaken and then kept at room temperature for 10 min; 1 µL sample was injected into the GC/MS system.

RESULTS AND DISCUSSION

Method development: The developed method for the assay of atenolol was based on its chemical properties. The column and acquisition parameters were chosen to be a starting point for the development of method. Atenolol is a polar molecule, therefore, the capillary column coated with 5 % phenyl and 95 % dimethylpolysiloxane is a good choice for separation of atenolol.

The GC/MS parameters used in the development of method were based on the boiling point. The injection port and detector temperature was set to 280 ºC for GC/MS. Different temperature programs were investigated for method. The end of this investigation, the temperature program of the GC/MS was as follows; initial temperature was 150 ºC, held for 1 min, increased to 220 ºC at a rate of 20 ºC/min held for 1 min and finally to 300 ºC at a rate of 10 ºC/min and held for 1 min. The injector volume was 1 mL in splitless mode.

N-Methyl-N-trimethylsilyl-trifluoroacetamide is an effective trimethylsilyl (TMS) donor. N-Methyl-N-trimethylsilyl-trifluoroacetamide reacts to replace labile hydrogens on a wide range of polar compounds with a TMS group and is used to prepare volatile and thermally stable derivatives for GC/MS[13]. To increase the performance of the gas chromatographic seperation, atenolol and internal standard were derivatized using MSTFA (Fig. 1). The secondary amine (-NH) and hydroxy (-OH) groups were converted to the corrosponding silyl (-N-TMS) and (-O-TMS) groups.

The effect of time and temperature on the reaction was investigated. To confirm the complete derivatization of atenolol and internal standard, since only one peak appears on the chromatogram, each compound was derivatized and analyzed separately. After establishing the optimum reaction conditions, the compounds were mixed together and then derivatized in order to perform a simultaneous analysis. To 100 µL of 1000 ng/mL atenolol solution and 100 µL of MSTFA solution were added and reacted at room temperature, 50 and 75 ºC for 5, 10 and 20 min, respectively. The resulting samples were quantitated by GC/MS system. The effect of the time and temperature was shown in Fig. 2.

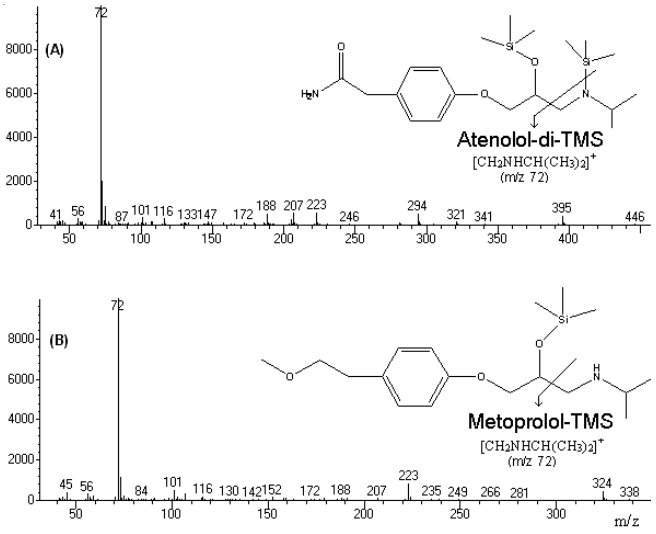

Fig. 1. MS spectra after derivatization of atenolol (A) and metoprolol (IS) (B) with MSTFA

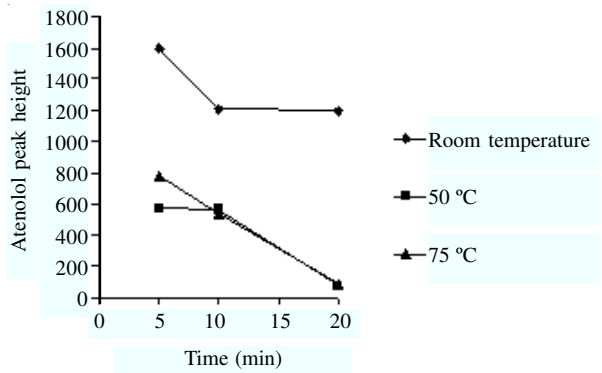

Fig. 2. Effect of reaction time and temperature on derivatization reaction

Method validation: The validation was carried out by establishing selectivity, linearity, intra- and inter-day precision, accuracy, recovery and sensitivity according to ICH guidelines for validation of the analytical procedures[12].

Selectivity: The selectivity of the assay was checked by comparing the chromatograms of six different batches of blank rabbit plasma with the corresponding spiked plasma. Each blank sample should be tested for interference and no endogenous interferences was encountered (Fig. 3a). The fragment ion $[CH_2NHCH(CH_3)_2]$ + (m/z 72) was used for quantitation of atenolol and internal standard. The retention time of atenolol-di-TMS and IS-TMS in rabbit plasma was *ca.* 10.5 and 7.8 min (Fig. 3b,c).

Linearity: The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of an analyte in the sample. The concentrations of the spiked atenolol in rabbit plasma were 15, 25, 50, 100, 150, 200 and 250 ng/mL with constant concentration of internal standard (500 ng/mL). The calibration curves were established by plotting the ratio of the peak areas of atenolol and IS obtained after extraction of the spiked plasma sample. The equation of the calibration curve obtained from seven points was y = 0.013x - 0.005 with a

correlation coefficient (r = 0.998). The linear regression equation was calculated by the least squares method using Microsoft Excel® program and summarized in Table-1.

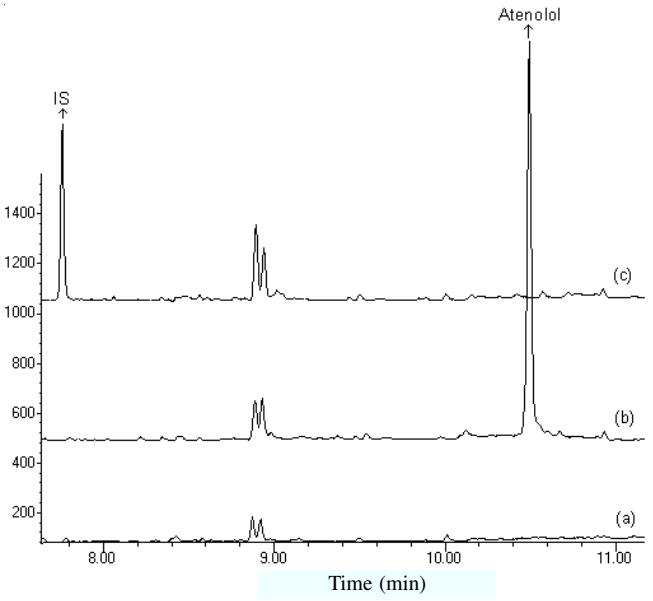

Fig 3. Typical SIM chromatogram of blank plasma (a), plasma spiked with 200 ng/mL atenolol (b) and 500 ng/mL metoprolol (IS) (c)

TABLE-1
LINEARITY OF ATENOLOL IN RABBIT PLASMA

Linearity (ng/mL)	15-250
Regression equation[a]	y = 0.013x - 0.005
RSD % of slope	4.81
RSD % of intercept	5.95
Correlation coefficent	0.998
RSD % of correlation coefficient	0.0198
Limit of detection (ng/mL)	5.0
Limit of quantification (ng/mL)	15

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

Precision and accuracy: Assay precision was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability during the same day and intermediate precision on different days (3 days) were evaluated with six replicates of quality control samples. The accuracy of analytical method was assessed as the percentage relative error (RE %). The accuracy and precision of the method were evaluated with quality control samples at concentrations of 35, 125 and 225 ng/mL. The intra- and inter-day accuracy and precision results are shown in Table-2. The intra- and inter-day precisions of the quality control samples were satisfactory with RSD less than 7.4 % and accuracy with relative error within ± 6.4 % (should be less than 15 according to CDER guidance for bio-analytical method validation)[14].

Limit of detection (LOD) and limit of quantification (LOQ): The limit of detection (LOD) is the lowest amount of atenolol in a sample which can be detected but not necessarily quantitated as an exact value. The limit of quantification (LOQ) is the lowest amount of atenolol which can be quantitatively determined with suitable precision.

The LOD and LOQ were studied to test the sensitivity of the method. The LOD defined as signal/noise = 3 in method (RSD > 20 % for LOD) was found to be 5 ng/mL. The LOQ defined as signal/noise = 10 in method (RSD > 10 % for LOQ) was found to be 15 ng/mL (Table-1). Both accuracy and precision of these values were well within the proposed criteria (RSD % < 20 %).

TABLE-2
PRECISION AND ACCURACY OF ATENOLOL IN RABBIT PLASMA

	Intra-day			Inter-day		
Added (ng/mL)	Found (mean ± SD[a])	Precision (%) RSD[b]	Accuracy[c]	Found (mean ± SD[a])	Precision (%) RSD[b]	Accuracy[c]
	Plasma pools[d]					
35	35.5±2.549	7.18	1.43	35.8±2.642	7.38	2.29
125	129.6±5.304	4.09	3.68	132.3±9.022	6.82	5.84
225	228.9±7.504	3.28	1.73	239.4±9.734	4.07	6.36

a: Standard deviation of six replicate determinations, RSD: relative standard deviation, b: Average of six replicate determinations, c: (Percentage relative error) (found-added)/added × 100, d: Plasma volume (0.5 mL)

Recovery: The recovery of an analyte is the extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method[15]. The liquid-liquid extraction was used for the sample preparation in this work. Several solvents (ethyl acetate, dichloromethane, acetonitrile, butanol and chloroform) were tested for the extraction. Finally, chloroform and butanol mixture (4:1, v/v) proved to be the most efficient in extracting atenolol from rabbit plasma. After extraction procedure, 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 kept at room temperature for 10 min. 1 µL sample was injected into the GC/MS system. Spiked plasma samples were prepared three time at all levels (15, 25, 50, 100, 150, 200 and 250 ng/mL) of the calibration graph of atenolol. The recovery of atenolol was determined by comparing the peak areas measured after analysis of spiked plasma samples with those found after direct injection of standard solutions at the same concentration levels. The extraction recoveries of atenolol from rabbit plasma were between 87.3 and 93.2 % as shown in Table-3.

TABLE-3
RECOVERY OF ATENOLOL IN RABBIT PLASMA

Added (ng/mL)	Found (mean ± SD[a])	Recovery (%)	RSD[b] (%)
15	13.1 ± 0.988	87.3	7.54
25	22.6 ± 1.342	90.4	5.94
50	44.9 ± 2.914	89.8	6.49
100	93.2 ± 4.884	93.2	5.24
150	138.6 ± 10.28	92.4	7.42
200	183.0 ± 11.84	91.5	6.47
250	221.5 ± 12.69	88.6	5.73

a: Standard deviation of three replicate determinations RSD: Relative standard deviation, b: Average of three replicate determinations.

Matrix effect: The matrix effect was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample[14]. The matrix effect of atenolol was investigated by comparing peak area of atenolol solutions with processed blank samples reconstituted with atenolol solutions. The blank plasmas used in this study were from six different batches of rabbit plasma. If the ratio < 85 % or > 115 %, a matrix effect was implied. The relative matrix effect of atenolol at three different concentrations (50, 150 and 250 ng/mL) was less than ± 11.1 % (Table-4). The results showed that there was no matrix effect of the analytes observed from the matrix of plasma in this study.

TABLE-4
MATRIX EFFECT EVALUATION OF ATENOLOL AND IS IN RABBIT PLASMA (n = 3)

Samples	Conc. level (ng/mL)	A (Mean ± SD)	B (Mean ± SD)	Matrix effect (%)
Atenolol	50	46.3±3.12	51.1±2.87	90.6
	150	139.7±9.47	157.2±8.34	88.9
	250	228.6±11.53	240.7±9.54	94.9
IS	500	457.9±13.45	513.1±10.28	89.2

A: Amount of atenolol and IS derivatized in blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution), B: Amount of atenolol and IS derivatized with MSTFA

Interference study: To test of the effect of the drugs [carvedilol, nebivolol, rofecoxib, medazepam, diazepam, diclofenac, disulfiram, estradiol valerate and medroxyprogesterone acetate] we spiked solutions of these drugs (1000 ng/mL) together with 1000 ng/mL atenolol solution in rabbit plasma, mixed and then extracted. The peak areas of the extracted sample solutions were measured. The tolerance was defined as the concentration of the added substance causing a relative error less than ± 3 %. In the case solutions of these drugs we did not notice any interference.

Stability: The stability of atenolol in rabbit plasma was studied under a variety of storage and handling conditions at low (75 ng/mL) and high (250 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 thawed at room temperature and kept at this temperature for 8 h. Freeze-thaw stability was checked through three cycles. Three aliquots at each of the low and high concentrations were stored at -20 ºC 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-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 ºC for 1 week. The accuracy of atenolol stability was obtained for the short-term temperature, freeze-thaw and long-term 97.6, 96.9 and 95.9 %, respectively. The stability results indicated that no significant degradation of atenolol in rabbit plasma was observed under the tested conditions.

Application to pharmacokinetic study of rabbits: The method was applied to quantify the plasma concentration of atenolol in a single-dose pharmacokinetic study conducted on six New Zealand white rabbits. The study was conducted in accordance with the animal ethical guidelines for investigations in laboratory animals and was approved by the Ethical Committee for Medical Experimental Research and Application Centre of Ataturk University. The rabbits are male which are 3.8-4.1 kg weights. The rabbits were housed with free access to food and water, except for the final 2 h before experimentation. After a single oral administration of 50 mg of atenolol (Tensinor tablet), 1.5 mL of blood samples were collected from the marginal ear vein at 0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0 and 12.0 h time-points into EDTA collection tubes. The blood was immediately centrifuged 6000 × g for 10 min at ambient temperature. The supernatant plasma layer was separated and analyzed for atenolol concentrations as described above. GC/MS chromatogram of rabbit plasma is shown in Fig. 4. Representative mean plasma concentrations *versus* time profiles following a single oral administration of atenolol six rabbits are presented in Fig. 5.

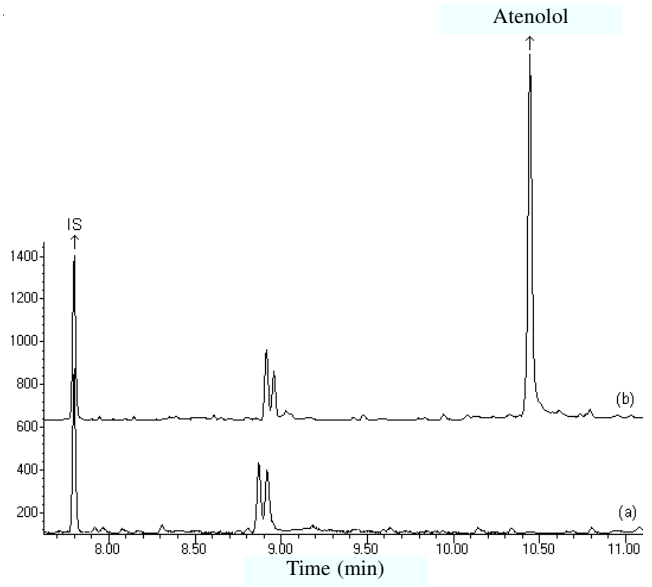

Fig. 4. Typical SIM chromatogram of plasma obtained from a rabbit at before (a) and after 3 h (b) oral administration of 50 mg atenolol

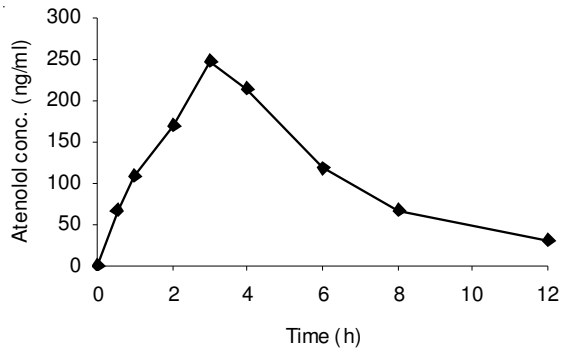

Fig. 5. Mean plasma concentration-time profile of atenolol in rabbits (n = 6) after application of tensinor tablet

The total area under the observed plasma concentration-time curve (AUC) was calculated by using the linear trapezoidal rule. The first order elimination rate constant (k_{el}) was estimated by the least square regression of the points describing the terminal log-linear decaying phase. $T_{1/2}$ was derived from k_{el}

($T_{1/2} = \ln 2/k_{el}$). The maximum observed atenolol concentration (C_{max}) and the time at which C_{max} was observed (T_{max}) were directly reported from the profile[16]. The main pharmacokinetic parameters of atenolol in six rabbits were calculated and summarized in Table-5.

TABLE-5
MEAN PHARMACOKINETIC PARAMETERS OF ATENOLOL FOR SIX RABBITS AFTER ORAL ADMINISTRATION OF TENSINOR TABLET (50 mg)

Parameters	Mean ± SD	RSD (%)
Maximum plasma concentration C_{max} (ng/mL)	248.1 ± 41.11	16.57
Time required for maximum plasma concentration T_{max} (h)	3.0 ± 0.577	19.23
Area under curve $AUC_{(0 \rightarrow 12\ h)}$ (ng/mL h)	1120.8 ± 204.43	18.24
Area under curve at infinite time $AUC_{(0 \rightarrow \infty)}$ (ng/mL h)	4966.9 ± 758.44	15.27
Plasma half life, $T_{1/2}$ (h)	3.47 ± 0.390	11.24

Pires de Abreu *et al.*[2] have reported 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. The LOQ and LOD of method were 25 and 10 ng/mL, respectively. Black *et al.*[11], have reported GC/MS method after pre-column derivatization with pentafluoropropionate for the analysis of β-blockers in human postmortem blood and urine.

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 preconversion 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. Also, the detection limits were lowered to pg levels by GC combined with MS[17].

The selectivity of GC/MS method has been demonstrated by the representative chromatograms for atenolol in rabbit plasma (Figs. 3 and 4). The retention time of atenolol in rabbit plasma is 10.5 min. The samples received from six different batches of blank plasma have also been tested and showed no significant interference at the retention time of compound of atenolol. The recovery of atenolol was achieved by developed liquid-liquid extraction procedure in rabbit plasma. Atenolol was extracted from rabbit plasma with a mixture of chloroform and butanol at basic pH. This solvent mixture gave an excellent recovery. The analytical recovery of atenolol from rabbit plasma averaged out to 90.46 %. Atenolol was extracted from blood and urine with a solid phase extraction procedure by Black *et al.*[11]. This method is also the most comprehensive method which can extract atenolol in a single extraction procedure.

Intra- and inter-day precision values for atenolol in rabbit plasma were less than 7.4 %. The LOD and LOQ were found to be 5 and 15 ng/mL, respectively. When this method is applied to plasma samples, its sensitivity was found to be adequate for pharmacokinetic studies. The present method has the following advantages over the reported method[6,10]. The LOD values of the reported methods were 10 and 27 ng/mL whereas the present method LOD was 5 ng/mL. Li *et al.*[9] have reported LC method with tandem mass detection for the analysis of atenolol in human plasma. Detection using LC/MS/MS would be more sensitive approach but is costly and not yet available for every laboratory. Additionally, this method was applied to six rabbits which had been given an oral tablet of 50 mg atenolol. The amount of atenolol was determined between 0 and 12 h in rabbit plasma.

Conclusion

In present work, a new, simple and sensitive GC/MS method has been developed. Also, GC/MS method is completely validated by using selectivity, stability, sensitivity, linearity, accuracy and precision parameters for determination of atenolol in rabbit plasma. The method was found to be linear over an analytical range of 15-250 ng/mL. Additional advantages of this method include small sample volume (0.5 mL), good extraction recovery from plasma and a readily available internal standard. The extraction and derivatization procedures in this study are simple. Therefore, the proposed method can be used as a therapeutic drug monitoring method in clinic to check the plasma concentration of atenolol in the patients with hypertension.

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