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DEVELOPMENT AND VALIDATION OF GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR DETERMINATION OF METOPROLOL IN RABBIT PLASMA

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ABSTRACT

This paper describes a gas chromatography-mass spectrometry (GC-MS) method with selected ion monitoring (SIM) for determination of metoprolol in rabbit plasma. Metoprolol and internal standard (IS) atenolol were extracted from plasma and cleaned up by using a single step liquid-liquid extraction. Derivatization was carried out using *N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA).* Calibration curves were linear over the concentration range 15-500 ng mL^{-1}. Intra- and inter-day precision values for metoprolol in rabbit plasma were less than 6.4, and accuracy (relative error) was better than 8.8%. The analytical recovery of metoprolol from rabbit plasma averaged out to 91.20%. The limits of detection (LOD) and quantification (LOQ) of metoprolol were 5.0 and 15 ng mL^{-1}, respectively. Also the developed and validated GC-MS method was successfully applied to a pharmacokinetic study of metoprolol in New Zealand white rabbits.

KEYWORDS

Metoprolol, Gas chromatography-mass spectrometry (GC-MS), Validation and Pharmacokinetics

INTRODUCTION

β-blockers are clinically important drugs and are used in the treatment of disorders such as hypertension, angina pectoris and arrhythmia. Metoprolol, 1-(isopropylamino)-3-[p-(2-methoxyethyl)phenoxy]-2-propanol, is a relatively

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selective β-1 adrenoceptor antogonist that has been used extensively for more than 25 years to treat such cardiovascular disorders as hypertension, arrhythmia and heart failure[1,2].

Several methods have been reported for determination of metoprolol including gas chromatography-mass spectrometry (GC-MS)[3-5], high-performance liquid chromatography (HPLC)[6-10], LC-MS[11-13] and LC-MS-MS[14].

On extensive survey of literature, no method is reported till date for determination of metoprolol by GC-MS in rabbit plasma. Therefore, we report a GC-MS method for determination of metoprolol after a derivatization procedure in rabbit plasma using IS methodology. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to International Conference on Harmonization guidelines[15].

The advantages of present method include simple and single step extraction procedure using inexpensive chemicals and short run time. Also, this method was used to assay the metoprolol in plasma samples obtained from six rabbits which had been given an oral tablet of Problok (100 mg metoprolol).

MATERIALS AND METHODS

(i) Chemicals and reagents:

Metoprolol tartrate, *N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA),* ethylacetate, dichloromethane, acetonitrile, diethylether and chloroform were purchased from Sigma-Aldrich (St. Louis, MO, USA). Atenolol as internal standard (IS) was kindly donated from Abdi Ibrahim Pharmaceutical Industry (Istanbul, Turkey). Problok tablet (100 mg metoprolol tartrate) was obtained Terra Pharmaceutical Industry (Istanbul, Turkey).

(ii) Apparatus and analytical conditions:

Chromatographic analysis was carried out on an Agilent 6890N gas chromatography system equipped with 5973 series mass selective detector, 7673 series autosampler and 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^{-1}. The injector and detector temperatures were 280 ^{0}C. The MS detector parameters were transfer line temperature 280 ^{0}C, solvent delay 3 min and electron energy 70 eV. The oven temperature program was held at 150 ^{0}C for 1 min, increased to 220 ^{0}C at a rate of 20 ^{0}C min^{-1} for 1 min and then increased to 300 ^{0}C at a rate of 10 ^{0}C min^{-1} for 1 min.

(iii) Preparation of stock and standard solutions:

10 mg metoprolol and IS were 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^{-1}). After, 5000 ng mL^{-1} standard solution was prepared by diluting with acetonitrile appropriate volumes of 20 μg mL^{-1} stock solution and stored at -20 ^{0}C under refrigeration. IS working solution was prepared at final concentration of 2500 ng mL^{-1}. The working solutions were prepared by diluting the standard stock solution from 15 ng mL^{-1} to 500 ng mL^{-1}. Also, quality control (QC) solutions were prepared from stock solution at concentrations of 75, 250 and 450 ng mL^{-1} together with 250 ng mL^{-1} IS.

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(iv) Sample preparation and derivatization procedure:

A 0.5 mL blank plasma of New Zealand white rabbit was transferred to a 12 mL centrifuge tube. 0.1 mL of standard metoprolol solutions together with 0.1 mL IS solution (250 ng mL^{-1}) and 0.5 mL 1 M sodium hydroxide solution were added. After vortex mixing for 5 second, 3 mL of ethylacetate and diethylether was added (2:1, *v/v*), the mixture was vortexed for 30 second and then centrifuged at 3000 x g 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 delayed at room temperature for 10 min. 1 µL sample was injected into the GC-MS system.

RESULTS

1. Method development:

MSTFA is an effective trimethylsilyl (TMS) donor. *MSTFA* 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[16]. To increase the performance of the gas chromatographic seperation, metoprolol and IS were derivatized using *MSTFA* (Figure 1). The secondary amine (-NH) and hydroxy (-OH) groups were converted to the corrosponding silyl (-N-TMS) and (-O-TMS) groups.

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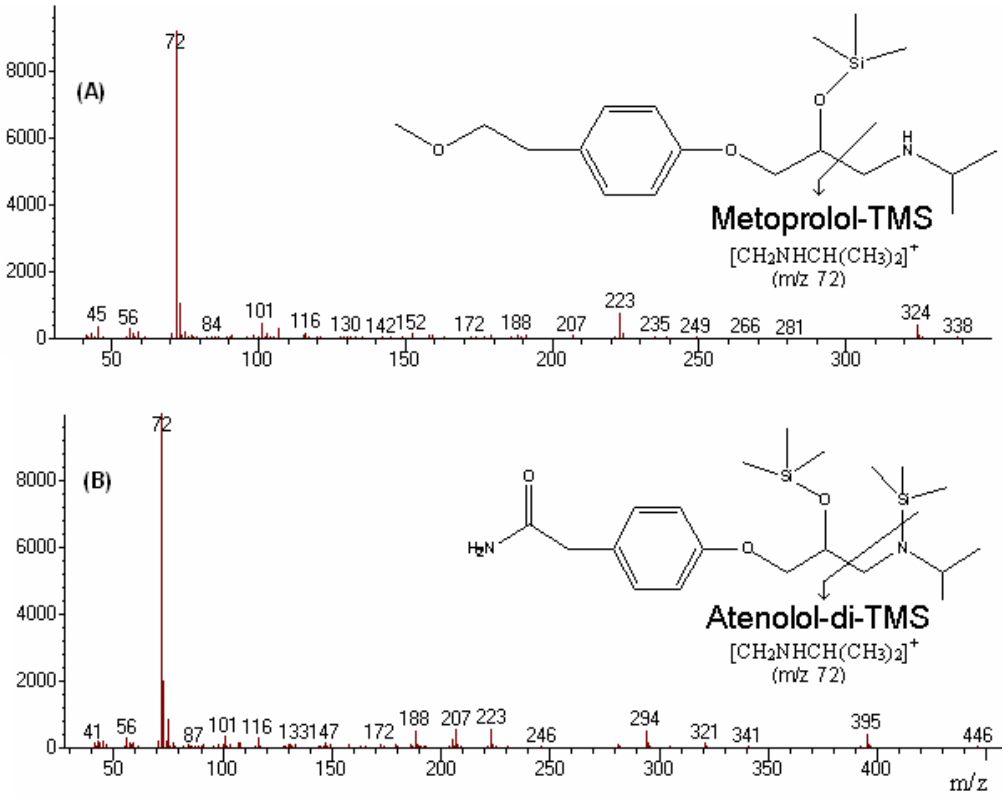

Figure 1

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

The effects of time and temperature on the reaction were investigated. To confirm the complete derivatization of metoprolol and IS, 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^{-1} metoprolol solution and 100 µL of *MSTFA* solution were added and reacted at room temperature, 50 and 75 ^{0}C for 5, 10 and 20 min. The resulting samples were quantitated by GC-MS system. The effect of the time and temperature was shown in Figure 2.

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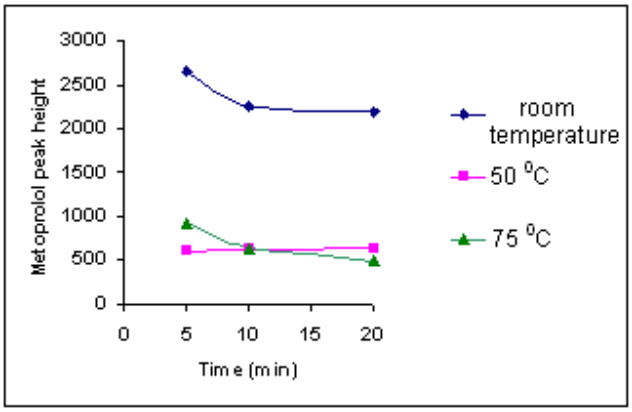
Figure 2

The effect of reaction time and temperature on derivatization reaction.



2. Validation of the method:

The validation was carried out by establishing specifity, linearity, intra- and inter-day precision, accuracy, recovery and sensitivity parameters according to ICH[15] and Center for Drug Evaluation and Research[17] guidance for Bio-analytical Method Validation.

2.1. Specificity:

The specificity criterion tries to demonstrate that the result of the method is not affected by the presence of interferences[18]. The specificity of method was determined by checking the chromatograms obtained from blank plasma samples, and no endogenous interferences were encountered. The fragment ion $[CH_2NHCH(CH_3)_2]^+$ (m/z 72) was used for quantification of metoprolol and IS. The retention time of metoprolol in rabbit plasma was approximately 7.8 min with good peak shape (Figure 3).

2.2. 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 metoprolol in rabbit plasma were 15, 50, 100, 200, 300, 400 and 500 ng mL^{-1} with constant concentration of IS (250 ng mL^{-1}). The calibration curves were established by plotting the ratio of the peak areas of metoprolol and IS obtained after extraction of the spiked plasma sample.

The equation of the mean calibration curve obtained from seven points was y=0.052x + 0.2134 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.

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Figure 3

Typical SIM chromatogram of blank plasma (a), plasma spiked with 200 ng mL^{-1} metoprolol (b) and 250 ng mL^{-1} atenolol (IS) (c).

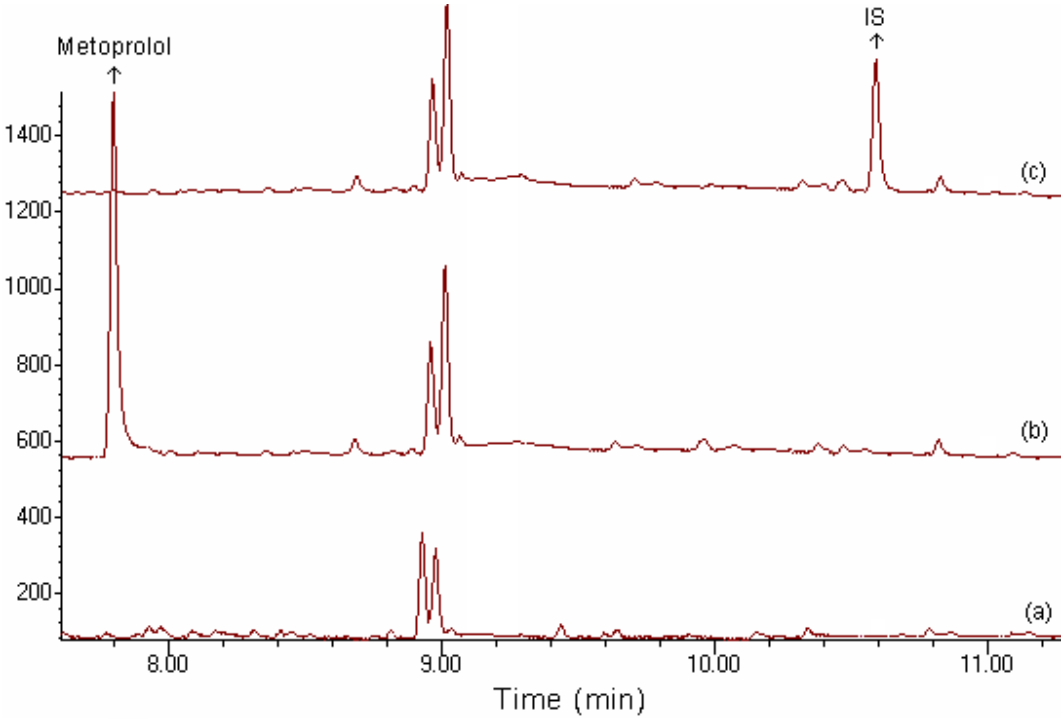

Table 1

Linearity of metoprolol in rabbit plasma.

Linearity (ng mL^{-1})	15-500
Regression equation[a]	y=0.052x+0.2134
RSD% of slope	3.12
RSD% of intercept	4.83
Correlation coefficient	0.998
RSD% of correlation coefficient	0.0182
Limit of detection (ng mL^{-1})	5.0
Limit of quantification (ng mL^{-1})	15

[a]Based on three calibration curves, y: peak-area ratio, x: metoprolol concentration (ng mL^{-1}), RSD: Relative standard deviation.

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2.3. 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 QC samples. The accuracy of analytic method was assessed as the percentage relative error. The accuracy and precision of the method were evaluated with QC samples at concentrations of 75, 250 and 450 ng mL^{-1}. The intra- and inter-day accuracy and precision results are shown in Table 2.

The intra- and inter-day precision of the QC samples were satisfactory with RSD less than 6.4% and accuracy with relative error within ± 8.8% (should be less than 15 according to CDER guidance for Bio-analytical Method Validation).

2.4. Sensitivity [limits of detection (LOD) and quantification (LOQ)]:

The LOQ is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOD is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. 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 5.0 ng mL^{-1}. The LOQ defined as signal/noise=10 in method (RSD>10% for LOQ) was found to be 15 ng mL^{-1} (Table 1). Both accuracy and precision of these values were well within the proposed criteria (RSD% < 20%).

2.5. 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[19]. The liquid-liquid extraction was used for the sample preparation in this work. Several solvents (ethylacetate, diethylether, dichloromethane, acetonitrile, butanol and chloroform) were tested for the extraction. Finally, ethylacetate and diethylether mixture (2:1, *v/v*) proved to be the most efficient in extracting metoprolol from rabbit plasma. Spiked plasma samples were prepared in three time at all levels (15, 50, 100, 200, 300, 400 and 500 ng mL^{-1}) of the calibration graph of metoprolol.

Table 2

Precision and accuracy of metoprolol in rabbit plasma.

	Intra-day			Inter-day		
Added (ng mL^{-1})	**Found (mean± SD[a])**	**Precision % RSD[b]**	**Accuracy[c]**	**Found (mean ± SD[a])**	**Precision % RSD[b]**	**Accuracy[c]**
Plasma pools[d]						
75	81.53 ± 2.767	3.39	8.71	78.56 ± 2.948	3.75	4.75
250	256.7 ± 2.522	0.98	2.68	241.9 ± 15.48	6.39	-3.24
450	458.5 ± 6.869	1.49	1.89	461.8 ± 9.749	2.11	2.62

SD[a]: Standard deviation of six replicate determinations RSD: relative standard deviation

[b]Average of six replicate determinations Accuracy[c]: (% relative error) (found-added)/addedx100, [d] Plasma volume (0.5 mL).

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Table 3

Recovery of metoprolol in rabbit plasma.

Added (ng mL^{-1})	Found (mean ± SD[a])	% Recovery	% RSD[b]
15	13.35 ± 1.125	89.0	8.43
50	45.60 ± 2.941	91.2	6.45
100	93.40 ± 4.931	93.4	5.28
200	184.2 ± 8.049	92.1	4.37
300	266.1 ± 19.85	88.7	7.46
400	380.4 ± 21.79	95.1	5.73
500	443.0 ± 28.39	88.6	6.41

SD[a]: Standard deviation of three replicate determinations, [b]Average of three replicate determinations.

The recovery of metoprolol 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 metoprolol from rabbit plasma were between 88.6 and 95.1% as shown in Table 3.

2.6. 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[17]. The matrix effect of metoprolol was investigated by comparing amount of metoprolol solutions with processed blank samples reconstituted with metoprolol solutions. The blank plasmas used in this study were from six different batches of rabbit blank plasma. If the ratio <85% or >115%, a matrix effect was implied. The relative matrix effect of metoprolol at three different concentrations (50, 200 and 500 ng mL^{-1}) was less than ± 10.9% (Table 4).

The results showed that there was no matrix effect of the analytes observed from the matrix of plasma in this study.

2.7. Stability:

The stability of metoprolol in rabbit plasma was studied under a variety of storage and handling conditions at low (100 ng mL^{-1}) and high (500 ng mL^{-1}) 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. Freze-thaw stability (-20 ^{0}C in rabbit plasma) was checked through three cycles. Three aliquots at each of the low and high concentrations were stored at -20 ^{0}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 freze-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 ^{0}C for 1 week. The accuracy of metoprolol stability was obtained for the short-term temperature, freze-thaw and long-term 97.8, 96.2 and 95.4%, respectively. The stability results indicated that no significant degradation of metoprolol in rabbit plasma was observed under the tested conditions.

2.8. Interference study:

In order to assess the possible analytical applications of the proposed method, the effect of common drugs used for patients was studied by analyzing spiked rabbit plasma samples. The absence of interfering compounds was verified by comparison of chromatograms of plasma samples with those of calibration plasmas. To test of the effect of the drugs [carvedilol, nebivolol, mexiletine, rofecoxib, medazepam, diazepam, diclofenac, disulfiram, estradiol valerate and medroxyprogesterone acetate] we spiked solutions of

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these drugs (1000 ng mL^{-1}) together with 1000 ng mL^{-1} metoprolol 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.

2.9. Pharmacokinetic study of rabbits:

The method was applied to quantify the plasma concentration of metoprolol 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 is 4.8-5.2 kg weight. 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 100 mg of metoprolol (Problok tablet), 1.5 mL of blood samples were collected from the marginal ear vein at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 12 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 metoprolol concentrations as described above.

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 k_{el} 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 k_{el}[20].

GC-MS chromatogram of rabbit plasma after oral administration of Problok tablet (100 mg) is shown in Figure 4. Representative mean plasma concentration versus time profiles following a single oral administration of metoprolol is presented in Figure 5. Various pharmacokinetic parameters have been summarized in Table 5.

Table 4

Matrix effect evaluation of metoprolol and IS in rabbit plasma (n = 3).

Sample	Concentration level (ng mL^{-1})	A (mean ± SD)	B (mean ± SD)	% Matrix effect
Metoprolol	50	46.3 ± 3.12	51.1 ± 2.87	90.6
	200	184.7 ± 9.47	207.2 ± 8.34	89.1
	500	457.9 ± 13.45	513.1 ± 10.28	89.2
IS	250	228.6 ± 11.53	240.7 ± 9.54	94.9

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

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Figure 4

Typical SIM chromatogram of plasma obtained from a rabbit at before (a) and after 1.5 h (b), 4 h (c) oral administration of 100 mg metoprolol.

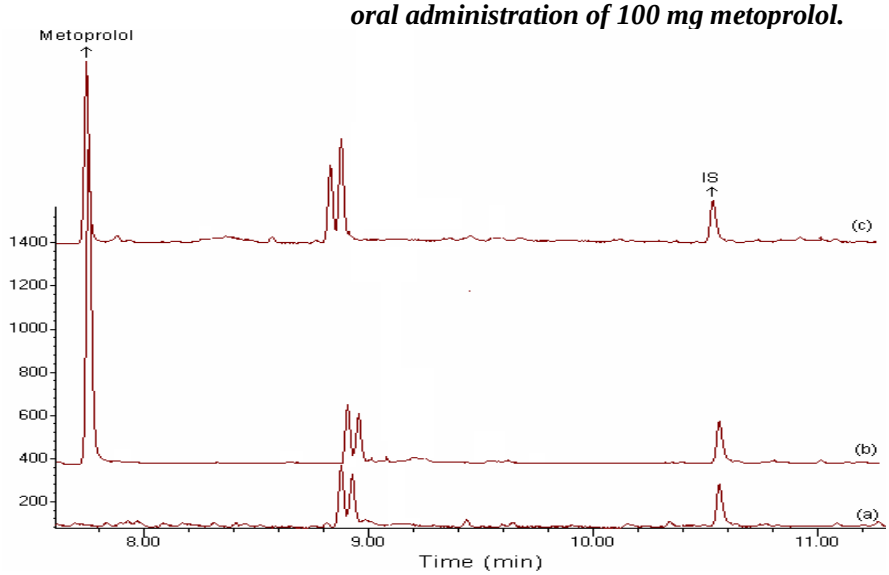

Figure 5

Mean plasma concentration-time profile of metoprolol in rabbits (n=6) after application of Problok tablet.

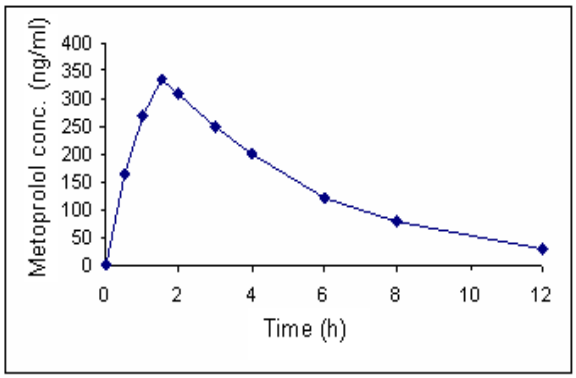

Table 5

Mean pharmacokinetic parameters of metoprolol for six rabbit after oral administration of Problok tablet (100 mg).

Parameters	Mean ± SD	% RSD
Maximum plasma concentration, C_{max} (ng mL^{-1})	335 ± 31.52	9.41
Time required for maximum plasma concentration, T_{max} (h)	1.50 ± 0.288	19.20
Area under curve, $AUC_{(0\rightarrow 12h)}$ (h ng mL^{-1})	1635.3 ± 240.56	14.71
Area under curve at infinite time, $AUC_{(0\rightarrow\infty)}$ (h ng mL^{-1})	5353.2 ± 710.37	13.27
Plasma half life, $T_{1/2}$ (h)	2.91 ± 0.409	14.05

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DISCUSSIONS

Kim et al.[3] have reported GC-MS method after pre-column derivatization with α-methoxy- α-(trifluoromethyl)phenylacetyl chloride as a chiral derivatizing agent for the determination of metoprolol in human urine. Angier et al.[4] have reported the determination of metoprolol with other β-blockers in biological fluids by GC-MS method after pre-column derivatization with pentafluoropropionic anhydride.

Albers et al.[6] have reported HPLC method with fluorescence detection for the analysis of metoprolol in human plasma. The calibration curve of the method was linear for metoprolol in the range 2.4-195.2 ng mL^{-1}. Precision throughout the whole working range was between 0.6 and 15.5%. Metoprolol recovery was determined at 73.0 ± 20.5, and the LOQ was 2.4 ng mL^{-1}.

Gowda et al.[14] have reported LC method with tandem mass detection for the analysis of metoprolol in human plasma. The calibration curve of LC-MS-MS method was linear for metoprolol in the range 5.0-500 ng mL^{-1}. Intra- and inter-day precision ranged from 4.82 to 8.42 and from 7.2 to 11.11% for metoprolol, respectively. The maximum recovery of metoprolol was 77.68%. The LOQ and LOD of method were found 5.0 and 1.0 ng mL^{-1}, respectively. Detection using LC-MS-MS would be a more sensitive approach but is costly and not yet available for every laboratory.

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 reguires 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. Also, the detection limits were lowered to pg levels by GC combined with MS[21].

The specificity of GC-MS method has been demonstrated by the representative chromatograms for metoprolol in rabbit plasma (Figures 3 and 4). The retention time of metoprolol in rabbit plasma is 7.8 min. The recovery of metoprolol was achieved by developed liquid-liquid extraction procedure in rabbit plasma. Metoprolol was extracted from rabbit plasma with a mixture of ethylacetate and diethylether solvents. This solvent mixture gave an excellent recovery. The analytical recovery of metoprolol from rabbit plasma averaged 91.20%. Metoprolol was extracted from urine with a solid phase extraction procedure by Chiu et al.[8] This method is also the most comprehensive method which can extract metoprolol in a single extraction procedure.

In statistical comparison ($p > 0.05$) with other methods in the literature[3, 6-8, 12, 14] the proposed method has indicated high accuracy, precision and recovery.

Metoprolol was analyzed in plasma, amniotic fluid and capillary blood by GC-MS according to Ervik et al.[22]. The minimum determinable concentration at standard deviation of 10% was 1 nmol L^{-1}. The present method has the following advantage over the reported method[23]. The LOQ of the reported method was 15.7 ng mL^{-1} whereas the present method LOQ was 15 ng mL^{-1}. When this method is applied to plasma samples, its sensitivity was found to be adequate for pharmacokinetic studies.

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CONCLUSION

In the present work, a simple and sensitive GC-MS method has been developed for the determination of metoprolol in rabbit plasma. Also, the method was completely validated by using sensitivity, stability, specificity, linearity, accuracy and precision parameters for determination of metoprolol in rabbit plasma. Additional advantages of this method include small sample volume (0.5 mL), good extraction recovery from plasma and a readily available internal standard. Also, the extraction and derivatization procedures in this study were simple. To our knowledge, this is the first description of metoprolol pharmacokinetics in rabbit plasma by GC-MS method in the literature. It can be very useful and an alternate to performing pharmacokinetic studies in determination of metoprolol for clinical use.

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