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Determination of metoprolol in rabbit plasma by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A simple, specific and sensitive high-performance liquid chromatography (HPLC) method has been developed for the determination of metoprolol in rabbit plasma. Separation of metoprolol and atenolol (internal standard) was achieved on a Ace C₁₈ column (5 μ m, 250 \times 4.6 mm i.d.) using fluorescence detection with λ_{ex} =276 nm and λ_{em} =296 nm. The mobile phase consisted of methanol-water (50:50, v/v) containing 0.1% trifluoroacetic acid (TFA). The analysis was performed in less than 5.0 min with a flow rate of 1ml/min. Calibration curve was linear over the concentration range 3-200ng/ml. Intra- and inter-day precision values for metoprolol in rabbit plasma were less than 5.0, and accuracy (relative error) was better than 4.8%. The analytical recovery of metoprolol from rabbit plasma averaged out to 95.7%. The limits of detection (LOD) and quantification (LOQ) of metoprolol were 1.0 and 3.0ng/ml, respectively. Also the developed and validated HPLC method was successfully applied to a pharmacokinetic study of metoprolol in New Zealand white rabbits. © 2010 Trade Science Inc. - INDIA

KEYWORDS

Metoprolol;
HPLC;
Liquid-liquid extraction;
Rabbit;
Pharmacokinetic study.

INTRODUCTION

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

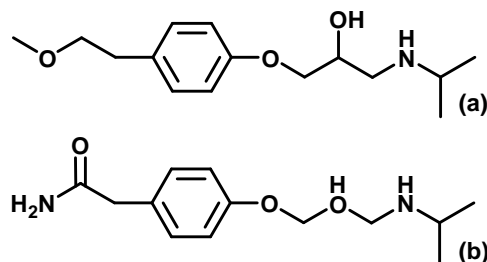


Figure 1 : Chemical structures of metoprolol (a) and atenolol, IS (b)

MS/MS^[14] in human plasma and other biological fluids.

On extensive survey of literature, no method is reported till date for determination of metoprolol by HPLC in rabbit plasma. Therefore, this report describes a sensitive and specific HPLC procedure with fluorescence

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detection for determining metoprolol in rabbit plasma using IS methodology. The method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to International Conference on Harmonization (ICH) 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 (100mg metoprolol).

EXPERIMENTAL

Chemicals and reagents

Metoprolol tartrate was obtained by 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). HPLC-grade organic solvents were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade. Distilled water was prepared as required by using aqua sMAX™ ultra, Young instrument (Korea) ultrawater purification system.

Apparatus and analytical conditions

A Hewlett-Packard series 200a HPLC system equipped with an HP 1046A programmable fluorescence detector and ChemStation software package was used (Hewlett-Packard, Wilmington, DE, USA). The HPLC mobile phase was composed of methanol-water (50:50, v/v) containing 0.1% TFA. Separation was achieved using an Ace C₁₈ column (5μm, 4.6×250mm i.d.) with a guard column (4mm×3mm i.d., Phenomenex) packed with the same material at a flow rate of 1ml/min. The eluent was monitored by fluorescence detection at 276 nm (excitation) and 296nm (emission).

Preparation of stock and standard solutions

5 mg metoprolol and IS were weighed, transferred 500ml volumetric flask, differently. 250ml methanol was added and the flask was sonicated. The flask was filled to volume with methanol (10μg/ml). After, 5000ng/ml standard solution was prepared by diluting

with methanol appropriate volumes of 10μg/ml stock solution and stored at -20°C under refrigeration. The working solutions were prepared by diluting the standard stock solution from 3.0ng/ml to 200ng/ml. Also, quality control (QC) solutions were prepared from stock solution at concentrations of 75, 250 and 450ng/ml together with 200ng/ml IS.

Extraction procedure

A 0.5ml blank plasma of New Zealand white rabbit was transferred to a 12ml centrifuge tube. 1.0ml of standard metoprolol solutions together with the IS solution (20μl, 10μg/ml) and 0.5 ml 1M 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×g for 7 min. The organic layer was transferred into another another 5ml tube and evaporated to dryness under stream of nitrogen gas at 40°C. The residue was reconstituted in 1ml methanol, and a 20μl aliquot was injected into the HPLC system.

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 3.8-4.1kg weight. After a single oral administration of 100mg of metoprolol (Problok tablet), 1.5 ml of blood samples was collected from the marginal ear vein at at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 12 h time-points into EDTA collection tubes. The blood was centrifuged 6000×g for 5 min. The plasma was separated and analyzed for metoprolol as described above.

RESULTS AND DISCUSSION

Method development and optimization

Method development was focused on the optimization of column detection, sample preparation and chromatographic separation. Reversed-phase column (C₁₈) can be used for the separation of non-ionic as well as ion forming non-polar to medium polar substances while normal phase chromatography can be used for the separation of non-ionic and/or non-polar sub-

stances. Majority of the ionizable pharmaceutical compounds can be very well separated on C_{18} ^[16]. Thus, metoprolol can be satisfactorily separated by reversed phase chromatography.

Several tests were performed for optimizing the components of mobile phase in order to achieve good chromatographic peak shape and resolution. The test results showed that the solvent system of water could improve the peak shapes of metoprolol. Good separation of target compounds and short run time were obtained using a mobile phase system of methanol-water (50:50, v/v) containing 0.1% TFA. The retention time of metoprolol (4.7 min) was quite short than that studied by Fang et al.^[2] and Chiu et al.^[8]. On the other hand, the mobile phase in the proposed method methanol-water instead of buffered systems is used in previously reported HPLC methods^[6,8]. Therefore, flushing of the column after analysis is not required.

Representative chromatograms of (a) blank rabbit plasma, (b) the rabbit plasma spiked with atenolol (100ng/ml) and IS (200ng/ml) and (c) the rabbit plasma obtained at 2 h after 100mg metoprolol were given in figure 2. There is no interference in the chromatogram of drug-free plasma.

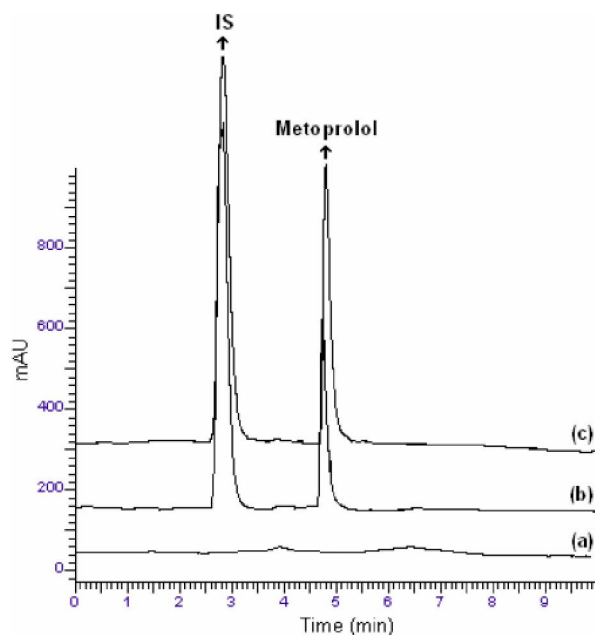


Figure 2 : Representative chromatograms of (a) blank rabbit plasma, (b) the rabbit plasma spiked with metoprolol (100ng/ml) and IS (200ng/ml), (c) the rabbit plasma obtained at 2 h after 100mg metoprolol

Validation of the method

Specificity

The specificity of method was determined by checking the chromatograms obtained from blank plasma samples, and no endogenous interference was encountered. Blank rabbit plasma was spiked with some drugs (carvedilol, nebivolol, ibuprofen, naproxen, mexiletine, rofecoxib, medazepam, diazepam, disulfiram, estradiol valerate and medroxyprogesterone acetate). The retention times for these drugs were determined and found not to interfere with metoprolol and IS.

Linearity

The calibration curve was 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 calibration curve obtained from seven points was $y=0.2984x - 0.0264$ with a correlation coefficient ($r=0.9985$). This method is as good as to that reported in the other papers^[3,5,7-9].

Precision and accuracy

Intra-day and inter-day precision were determined repeatability during the same day and intermediate precision on three different days were evaluated with six replicates of QC samples. The accuracy of this analytic method was assessed as the percentage relative error (% RE).

The method indicated very good precision and accuracy. Intra- and inter-day precision and accuracy for metoprolol in plasma are shown in TABLE 1. The intra- and inter-day precisions were measured to be within

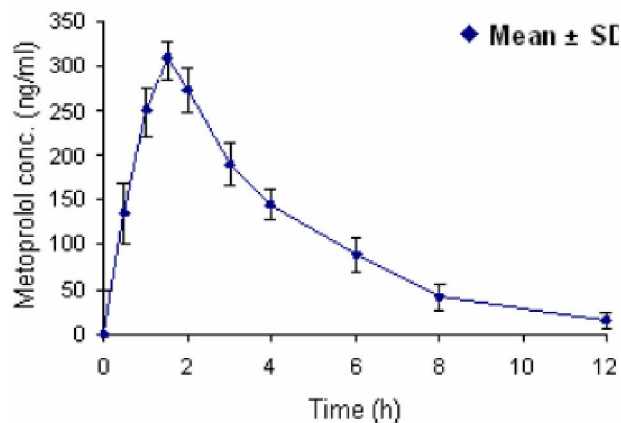


Figure 3 : Mean plasma metoprolol concentration-time profile for six rabbits after 100mg metoprolol

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TABLE 1 : Intra-day and inter-day precision and accuracy of metoprolol in plasma (n=6)

Sample	Concentration (ng/ml)		RSD%	RE%
	Added	Found (Mean ± SD)		
Plasma	12.5	11.92±0.223	1.87	-4.64
Intra-day	75	71.47±1.251	1.75	-4.71
	175	168.54±1.635	0.97	-3.69
Inter-day	12.5	12.09±0.596	4.93	-3.28
	75	78.02±2.956	3.79	4.03
	175	171.09±4.414	2.58	-2.23

1.87 and 4.93% for plasma. In statistical comparison ($p > 0.05$) with other methods in the literature^[3,6-8,12,14] the proposed method has indicated high accuracy and precision.

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

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 1.0ng/ml. The LOQ defined as signal/noise=10 in method (RSD>10% for LOQ) was found to be 3.0µg/ml. Both accuracy and precision of these values were well within the proposed criteria (RSD% < 20%). The proposed method has a detection and quantification limits of 3 and 5ng/ml for plasma which are as good or superior to that reported in the other papers^[5,7].

Recovery

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 95.4 and 96.3% as shown in TABLE 2. The mean recovery is better for plasma than those of the studies reported by Albers et al.^[6] Chiu et al.^[8] and Johnson et al.^[11].

Stability

The stability of metoprolol in rabbit plasma was studied under a variety of storage and handling conditions at low (12.5ng/ml) and high (175ng/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

TABLE 2 : Recovery of metoprolol in plasma (n=6)

Sample	Concentration (ng/ml)		Recovery %	RSD%
	Added	Found (Mean ± SD)		
Plasma	12.5	11.92±0.223	95.4	1.87
	75	71.47±1.251	95.3	1.75
	175	168.54±1.635	96.3	0.97

room temperature and kept at this temperature for 8 h. Freze-thaw stability (-20°C in rabbit plasma) 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 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°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.

Pharmacokinetic analysis

The proposed method was used to determine the plasma concentration of metoprolol. 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 elimination rate constant (k_{el}) and adding the result to the AUC_{0-t} . The elimination rate constant was calculated by the least squares regression using the last five time points of each curve. The apparent elimination half-life was the quotient of the natural logarithm of 2 and the elimination rate constant^[17].

Representative mean plasma concentrations versus time profiles following a single oral administration of atenolol to six rabbits are presented in figure 3. The main pharmacokinetic parameters of metoprolol were shown in TABLE 3.

CONCLUSION

In the proposed work, a new and sensitive HPLC

TABLE 3 : Mean pharmacokinetic parameters of metoprolol for six rabbits after Problok tablet (100mg)

Parameter	(Mean ± SD)	% RSD
Maximum plasma concentration C _{max} (ng/ml)	311.1±24.46	7.86
Time required for maximum plasma concentration T _{max}	1.50±0.216	14.40
Area under curve AUC _(0-12 h) (ng/ml h)	1610.2±32.42	20.13
Area under curve at infinite time AUC _(0-∞) (ng/ml h)	1942.3±41.17	21.19
Plasma half life (T _{1/2}) (h)	2.75±0.462	16.80

method has been developed and validated for determination of metoprolol in rabbit plasma. The stability studies showed that metoprolol in plasma were stable during short-term periods for sample preparation and analysis. Additional advantages of this method include small sample volume (0.5ml) and good extraction recovery in plasma. Also, the extraction procedure in this study were simple. Therefore, the method can be very useful and an alternate to performing pharmacokinetic studies in determination of metoprolol for clinical use.

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