Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 1, 2015

Original Article

ESTIMATION OF METOPROLOL IN HUMAN PLASMA BY HPLC METHOD

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Received: 12 Nov 2014 Revised and Accepted: 10 Dec 2014

ABSTRACT

Objective: The current project was undertaken to develop and validate a simple method for estimation of metoprolol in human plasma using HPLC. Before implementing the method on human plasma it was developed from scratch and validated, further human plasma was spiked with drug to achieve considerable results.

Methods: From UV absorption studies, λmax of metoprolol was found to be 262 nm. The mobile phase consisted of acetonitrile-potassium phosphate (pH 3.0) buffer in the ratio of 60:40 v/v. Propanolol was used as working internal standard. Standardization of metoprolol was performed on Grace C_{18} ODS (250 mm X 4.6 mm) reversed-phase column packed with 5 µm spherical ODS packing. Isocratic elution with acetonitrile-potassium phosphate (pH 3.0) buffer (60:40 v/v) was performed at a flow rate of 1.0 ml/min at ambient temperature. Shimadzu SPD 10 AT VP variable wavelength detectors and injection loop of 50 µl were used and metoprolol detection was carried out at 262 nm. The same method was implemented on human plasma spiked with the drug.

Results: Retention time of metoprolol was found to be 4.07±0.02 min and propranolol was found to be 3.98±0.05 min and when the plasma was spiked with the drug retention time of human plasma was 2.20±0.01 min, retention time of internal standard (Propranolol) was 3.89±0.05 min and retention time of metoprolol was 4.14±0.02 min respectively. The method developed was found to comply with standards LOD (Limit of Detection), LOQ (Limit of Quantification), Precision and Accuracy (Intra- and Inter-day variation), Specificity, Selectivity and Recovery/Extraction efficiency. Various validation parameters were analyzed and found to be complying well within the range.

Conclusion: Thus it can be concluded that the method developed is compatible for the estimation of the drug in human plasma and the same can be utilized to estimate the concentrations of the drug in patients on metoprolol therapy.

Keywords: Metoprolol, HPLC, Human plasma, Retention time.

INTRODUCTION

Metoprolol is a cardio selective β1-adrenergic blocking agent used for acute myocardial infarction (MI), heart failure, angina pectoris and mild to moderate hypertension. It may also be used for supraventricular and tachyarrhythmias and prophylaxis for migraine headaches[1**]**. Metoprolol is structurally similar to bisoprolol, acebutolol and atenolol in that it has two substituents in the *para* position of the benzene ring. The β1-selectivity of these agents is thought to be due in part to the large substituents in the *para* position. At low doses, metoprolol selectively blocks cardiac β1-adrenergic receptors with little activity against β2-adrenergic receptors of the lungs and vascular smooth muscle[2-4]. Receptor selectivity decreases with higher doses. It has a short half life but its β adreno receptor blocking effect is long lasting.

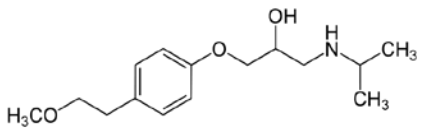

Fig. 1: Structure of Metoprolol

It is reported that, the incidence of sudden cardiac death peaks during the early morning hours when there is a rapid withdrawal of vagal tone and an increase of sympathetic tone.[5-6] In multiple clinical trials, β blockers have been found to decrease the incidence of stroke and overall cardiovascular mortality. To provide maximal benefit, in particular, to blunt the early-morning surge in blood pressure associated with the increased circadian incidence of sudden cardiac death, the more cardioselective and longer-acting agents of this drug class are preferred.[7-11]Metoprolol can be used in the above mentioned scenario judiciously with the knowledge of its chronokinetics and by adjusting the dosing schedule to achieve better efficacy and minimal adverse effects. To interpret the chronokinetics of any drug, its concentration in the given time has to be estimated in the plasma samples and hence this study was taken up to develop a simple and precise method for the analysis of metoprolol in human plasma. As not many studies have been carried out for simple estimation of the drug in human plasma using HPLC (UV) this study will be a tool for the researchers to plan their work on metoprolol concentrations.

High performance liquid chromatographic method can be used to estimate the amount of drug present in the plasma at given time and to optimize the dosing schedule.[12-15] A method was developed for estimation of metoprolol succinate in human plasma. This method was simple, accurate and precise. Separation of the component was achieved using Grace C_{18} reversed-phase column (250 mm X 4.6 mm, 5 µm). Isocratic elution was carried out using acetonitrile-potassium phosphate buffer (60:40 v/v) and propanolol was used as the internal standard. Detection was completed using UV detector at 262 nm.

MATERIALS AND METHODS

U. V. absorption studies of metoprolol

Determination of absorption spectra and absorption maxima

The U. V. absorption spectra and absorption maxima (**λ_{max}**) for metoprolol was determined by scanning a known concentration of metoprolol solution on a U. V-visible spectrophotometer at the wavelength range of 200 nm to 400 nm and the absorption maxima (**λmax**) of metoprolol was found to be 262 nm.

Estimation of metoprolol by HPLC

Prior to the standardization and validation of the reported method of estimation of metoprolol, a trial run was given in order check for the type of peak that may be obtained.

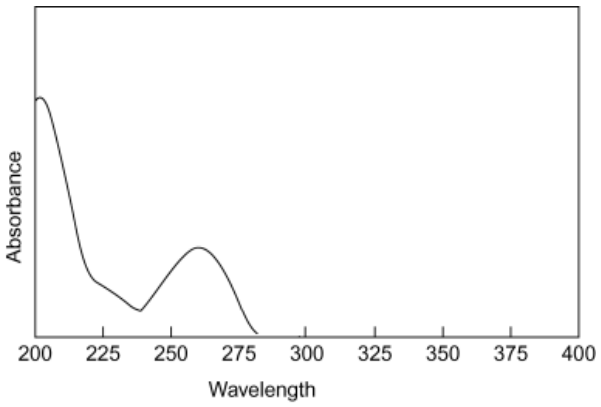

Fig. 2: Absorption spectrum of metoprolol

Equipment: High performance liquid liquid chromatography equipped with Shimadzu SPD 10 AT VP variable wavelength detector

Column: C_{18} ODS 2, 250 X 4.6 mm, reversed-phase column packed with 5 µm spherical ODS packing.

Buffer: 0.05 M potassium dihydrogenphosphate containing 1 ml triethylamine and adjusted to pH 3.0 with orthophosphoric acid.

Mobile Phase: Acetonitrile-potassium phosphate (pH 3.0) buffer (60:40 v/v)

Flow Rate: 1.0 ml/min

Temperature: Ambient

Wavelength: 262 nm

Injection volume: 50 µl

Preparation of the buffer & the mobile phase

The elution buffer was 0.05 M potassium dihydrogenphosphate containing 1 ml triethylamine and adjusted to pH 3.0 with orthophosphoric acid.

The mobile phase consisted of acetonitrile-potassium phosphate (pH 3.0) buffer in the ratio of 60:40 v/v. Required volume of the prepared buffer solution was taken in a 1000 ml volumetric flasks. To this, acetonitrile was added to make up the volume to 1000 ml. The resultant mobile phase was filtered using nylon membrane mobile phase filters (0.22 µm) and then degassed by sonicating it for 20 min. Chromatography was performed at room temperature (about 25ºc). The mobile phase was pumped at an isocratic flow rate of 1.0 ml/min at room temperature. The UV detection wave length was set at 262 nm. The eluent was detected using a UV/VIS detector set at 262 nm. Propranolol was used as working internal standard.

Chromatography

Standardization of metoprolol was performed on Grace C_{18} ODS 2, 250 X 4.6 mm, reversed-phase column packed with 5 µm spherical ODS packing. A guard column was positioned in the normal guard column position immediately in front of the analytical column. Isocratic elution was performed at a flow rate of 1.0 ml/min at ambient temperature. Shimadzu SPD 10 AT VP variable wavelength detector and injection loop of 50 µl were used and metoprolol detection was carried out at 262 nm. Retention time of metoprolol was found to be 4.07±0.02 min and propranolol were found to be 3.98±0.05 min respectively.

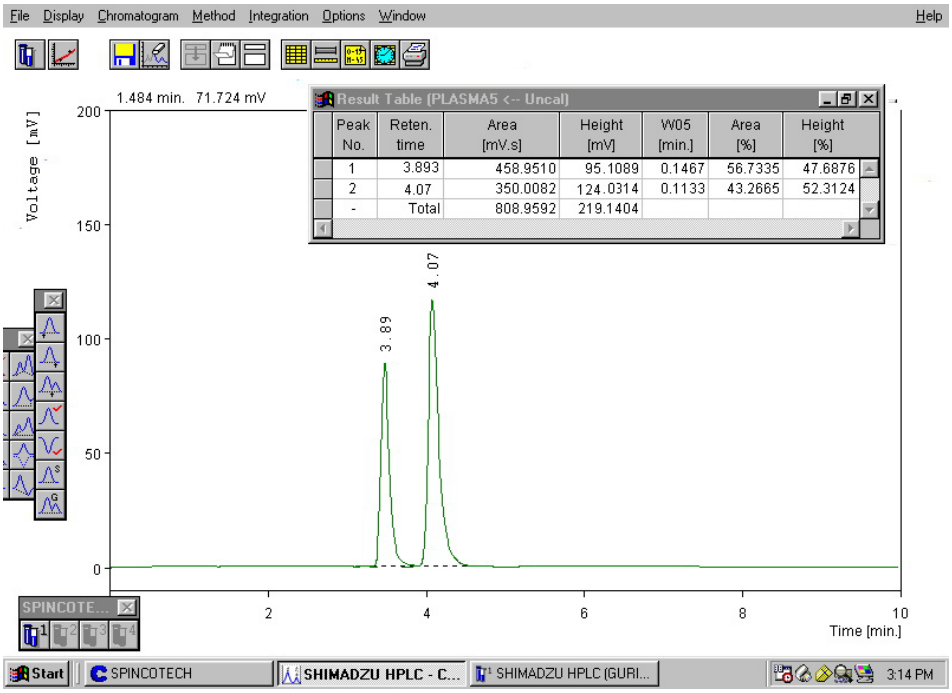

Fig. 3: Chromatogram for propranolol and metoprolol

Sensitivity adjustment

Concurrently, during the standardization process of the mobile phase, a study was carried out to investigate the effect of change of sensitivity of the instrument on the elution and resolution (peak area) of the metoprolol peak. The sensitivities that were evaluated were 1.0, 0.1, 0.08, 0.04, 0.06 and 0.02 a. u. f. s. Peak area ratio of metoprolol was obtained best at 0.1 a. u. f. s.

Standardization of the method of estimation of metoprolol:

Involves the determination of the following;

- Sensitivity

1. LOD (Limit of Detection)
2. LOQ (Limit of Quantitation)

- Accuracy & Precision (Intra-day variation)
- Absolute & relative recovery

Preparation of stock solution

10 mg of metoprolol was accurately weighed and was thoroughly and completely dissolved in mobile phase to obtain a concentration of 1000 µg/ml. 1 ml of the above solution was further diluted with 10 ml with mobile phase to get a concentration of 100µg/ml.

Preparation of working standard

Aliquots of 0.1 ml, 0.2 ml upto 5.0 ml were withdrawn from the stock solution and individually were diluted to 10 ml with mobile phase to get series of solutions of concentrations of 1µg, 2µg upto 50µg. The resultant sample solutions were filtered using sample filters (0.22 µm), sonicated for 10 min and then injected.

Similarly, a further dilution of the initial working internal standard (Propranolol) was carried out in order to study the lower concentrations of metoprolol. Aliquots of 0.002 ml, 0.005 ml, 0.010 ml upto 1.0 ml were withdrawn from the working standard (10 µg/ml) and individually diluted to 10 ml with mobile phase to get series of solutions of concentrations of 2 ng, 5 ng upto 1 µg. The resultant sample solutions were filtered using sample filters (0.22 µm), sonicated for 10 min and then injected.

Estimation of metoprolol in human palsma

Next step was standardization of the above mentioned method for estimation of metoprolol in human plasma. The technique involves spiking the pure drug of metoprolol to the plasma samples obtained from healthy human volunteers. Following this the standard calibration curve of metoprolol, as peak area (mV) Vs concentration (mcg/ml), was plotted.

Preparation of the buffer

The elution buffer was 0.05 M potassium dihydrogenphosphate containing 1 ml triethylamine and adjusted to pH 3.0 with orthophosphoric acid. The mobile phase consisted of acetonitrile-potassium phosphate (pH 3.0) buffer in the ratio of 60:40 v/v.

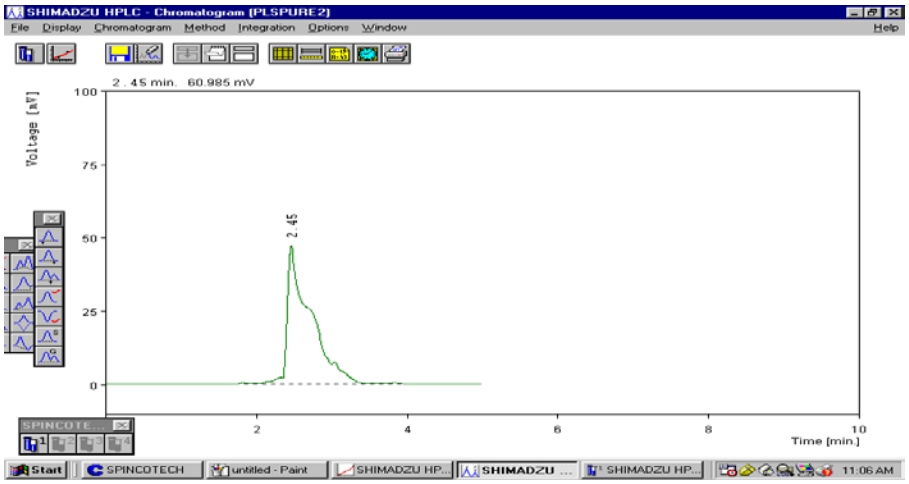

Fig. 4: Chromatogram of Blank Human Plasma

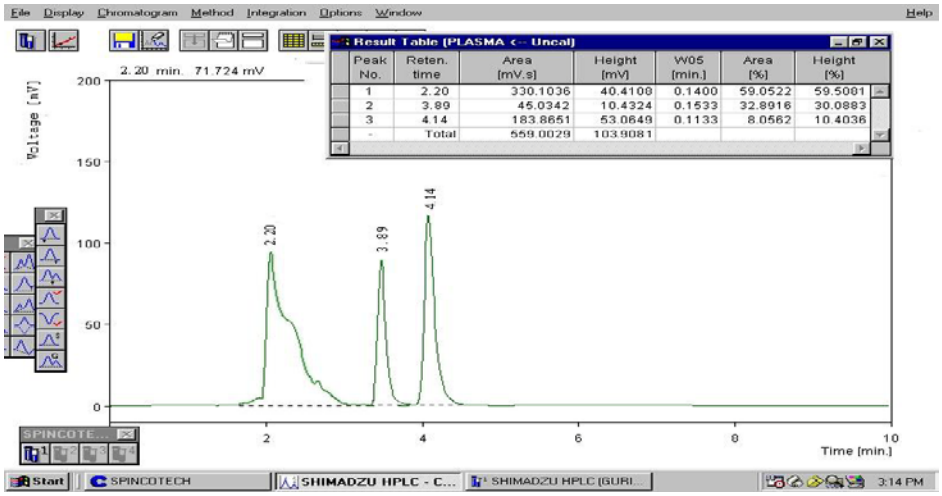

Fig. 5: Chromatogram of Human Plasma+ Propronolol (IS)+Metoprolol

Preparation of the plasma samples of metoprolol

Standard solutions (20 µl) of metoprolol (0.1-20 µg/ml) in mobile phase were added to 50 µl of drug-free plasma contained in a series of micro-centrifuge tubes. To this mixture, 20µl of the internal standard (20 µg/ml propronolol) was added followed by 25 µl of 20 % perchloric acid to precipitate the proteins. These plasma samples containing varying concentrations of metoprolol were vortex-mixed using a vortex mixer for 2 min and then centrifuged at 10,000 rpm for 5 min at 25°C using the cold-centrifuge. The clear supernatant obtained, after centrifugation, was transferred into clean and another series of micro-centrifuge tubes labeled respectively, the

supernatant so obtained after the second centrifugation was injected into the chromatographic system (HPLC). The chromatogram of plasma spiked drug is represented in fig. No: 05, where the retention time of human plasma is 2.20±0.01 min, retention time of an internal standard (Propranolol) was 3.89±0.05 min and retention time of metoprolol was 4.14±0.02 min respectively. Standard calibration curve was plotted as concentration (µg/ml) on X-axis and peak area on Y-axis and the linearity was determined (Table-1; Figure-06).

Table 1: Standard calibration curve of metoprolol in plasma by HPLC

S. No.	Volume (ml)	Concentration (µg/ml)	Peak area (± S. D)*
01	0.02	0.2	1.6402± 0.0019
02	0.04	0.4	2.5000± 0.0026
03	0.06	0.6	2.6021± 0.0218
04	0.08	0.8	3.5346± 0.0550
05	0.1	1.0	7.4485± 0.0212
06	0.2	2.0	10.0017± 0.0335
07	0.4	4.0	22.9711± 0.0440
08	0.6	6.0	42.7209± 0.0390
09	0.8	8.0	58.5621± 0.0321
10	1.0	10.0	78.5110± 0.0224
11	1.2	12.0	91.0061± 0.0493
12	1.4	14.0	109.1214± 0.0255
13	1.6	16.0	128.9038± 0.0213
14	1.8	18.0	158.5010± 0.0801
15	2.0	20.0	167.9095± 0.0032

Peak area ± S. D.*= average of three determinations

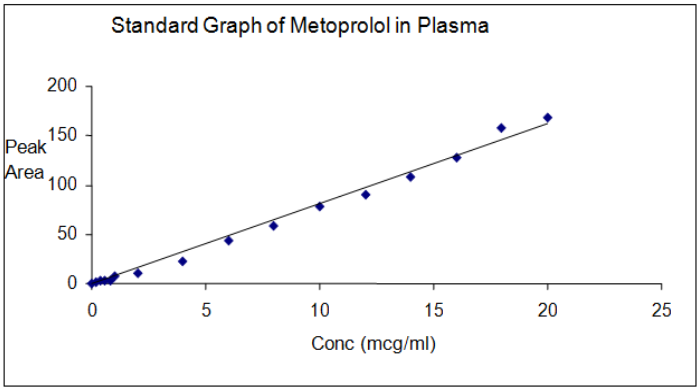

Fig. 6: Standard calibration curve of metoprolol in plasma by HPLC

RESULTS AND DISCUSSION

Method validation

Sensitivity

Limit of Detection [LOD]

For the determination of limit of detection (LOD), standard solutions (low concentrations) were prepared. LOD was found to be 0.02µg/ml (20 ng/ml) with the coefficient of variation less than 6%.

Limit of Quantification [LOQ]

Limit of quantification (LOQ)was determined by preparing a series of standard solutions (low concentrations), containing known amount of metoprolol. LOQ was found to be 0.03µg/ml (30ng/ml) with the coefficient of variation less than 6%.

Accuracy and Precision

The precision of the method was determined by the intraday variation studies. In the intraday studies, six repeated injections of standard solution of a particular concentration (10 µg/ml) were made. The response factor of drug peaks and percentage relative standard deviation (RSD) were calculated. From the data obtained, the precision of the HPLC method was determined.

Precision was expressed as the % Co-efficient of variation (%CV). The intra-day %CV ranged from 1.21 to 4.63 and 1.54 to 4.01 for Metoprolol and Propranolol. The inter-day %CV ranged from 0.98 to 2.44, and 1.12 to 6.90 for Metoprolol and Propranolol respectively.

The intra-day accuracy ranged from 100.01 to 102.33, and 101.01 to 103.87 for Metoprolol and Propranolol, whereas the inter-day accuracy ranged from 100.03 to 102.00, and 99.90 to 100.76 for Metoprolol and Propranolol respectively.

Absolute and relative recovery

The absolute recoveries of Metoprolol and Propranolol ranged from 98.4 ± 2.33 to 99.99±1.22, and 99.09± 4.00 to 100.83±1.23, whereas the relative recovery ranged from 100.57±32.33 to 101.98±1.56, and 101.90±1.85 to102.00±1.43.

Method validation for plasma samples spiked with metoprolol

Determination of linearity

Linearity was found in the concentration range of 0.2 – 20 µg/ml. The coefficient of correlation and percentage curve fitting was 0.9901 (R^2) and 99.01 % respectively.

Limit of Detection [LOD]

For the determination of limit of detection (LOD), plasma samples (low concentrations) were prepared in a manner similar to that prepared for the determination of linearity and calibration of the standard curve. The solutions were injected and chromatograms were obtained from which the LOD was determined. The analyte

concentration at which it could be reliably detected was 0.030µg/ml (30ng/ml)

Limit of Quantification [LOQ]

Limit of quantification (LOQ) was determined by preparing a series of plasma samples, containing known and varying concentrations of metoprolol. LOQ was determined from the chromatograms obtained. The smallest analyte concentration which could be accurately quantified was 0.05 µg/ml (50 ng/ml).

Precision and accuracy

The precision (RSD) values, for the within-batch and between-batch, for metoprolol was found to be 1.05 % and 2.3 % which is well within the acceptance criteria of not more than 2 %. The precision of the method/assay was found to be satisfactory. Similarly the accuracy values, for the within-batch and between-batch, for metoprolol were found to be 99.5 % and 101.2% which is well within the acceptance criteria of not more than 15 % (i. e. 85 % - 115 %). The accuracy of the method was found to be satisfactory.

Specificity

The pure mobile phase, i. e. filtered and degassed, was run for 15 min following which repeated injections of the pure mobile phase, without the drug, was done. No peaks were detected during the 20 minutes run of the pure mobile phase. Also, injections of the plain/placebo mobile phase (without the drug) did not give any peaks.

Selectivity

Five randomly selected blank human plasma samples were subjected to sample preparation in a manner similar to that prepared for the determination of linearity and calibration of the standard curve. These samples were chromatographed to determine the extent to which the plasma components may contribute to interference with the analyte. There were no interfering peaks present, in the randomly selected samples of blank human plasma that was chromatographed, at the retention times of the analytes.

Recovery/Extraction efficiency

Recovery or extraction efficiency of metoprolol was determined by comparing the mean peak areas of the neat reference solutions (unprocessed/unextracted), i.e. Standard solutions of metoprolol containing known amounts of metoprolol in mobile phase that is directly injected, to the mean peak areas of the extracted plasma samples containing same amounts of metoprolol. Recovery or extraction efficiency of metoprolol by this method was found to be 90.12 ±0.9 %.

CONCLUSION

Metoprolol is a β blocker ik which forms an important member of antihypertensive class of drugs widely used to treat hypertension more so in patients with cardiovascular complications. It has a short half life but its β adreno receptor blocking effect is long lasting.

The current work involved the development of a suitable method for the estimation of metoprolol through HPLC and validation of the same. This was done as a first step to quantify the amount of drug present in the human plasma and also to evaluate retention times of metoprolol when spiked in human plasma and to determine the feasibility of using the drug along with the plasma by using the method developed. Retention time of metoprolol was found to be 4.07±0.02 min and propanolol was found to be 3.98±0.05 min and when the plasma was spiked with the drug retention time of human plasma was 2.20±0.01 min, retention time of internal standard (Propranolol) was 3.89±0.05 min and retention time of metoprolol was 4.14±0.02 min respectively. Various validation parameters as discussed above were analyzed and were found to be complying well within the range. Therefore it can be concluded that the method developed is compatible for the estimation of metoprolol in human plasma and the same can be utilized to estimate the concentrations of the drug in patients on metoprolol therapy.

CONFLICT OF INTERESTS

None

ACKNOWLEDGEMENT

The authors wish to express gratitude to the Principapl & management of Al-Ameen College of Pharmacy, Bangalore for providing the necessary facilities to carry out the research work.

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