INTERNATIONAL JOURNAL OF CURRENT RESEARCH IN CHEMISTRY AND PHARMACEUTICAL SCIENCES
(p-ISSN: 2348-5213: e-ISSN: 2348-5221)
www.ijcrcps.com
DOI: 10.22192/ijcrcps Coden: IJCROO(USA) Volume 5, Issue 4 - 2018

Research Article

DOI: http://dx.doi.org/10.22192/ijcrcps.2018.05.04.011

Development and Validation of High Performance Liquid Chromatographic Method for the Determination of Celiprolol in human plasma

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Abstract

A rapid, specific and accurate high performance liquid chromatographic method for the determination of Celiprolol in human plasma**.** The extraction process involved a liquid-liquid extraction using a mixture of methyl-t-butyl ether and dichloromethane. Both Celiprolol and the internal standard were eluted under isocratic mode using a Ascentis 50 X 4.6 mm i.d, 5 µm column. The mobile phase composed a mixture of 10:90 % v/v 0.2% formic acid solution and acetonitrile at a flow rate of 1.0 mL/minute. A 50:50 splitterswas used to reduce the solvent entering into the ESI. The injection volume is 5 µL. The runtime of the method is 2 minutes. The method showed good linearity in the range of 0.25 – 35.06 µg/mL.The mean recovery of Celiprolol from all the quality control samples is 45.29 % with a % coefficient of variation of of 7.5 % and recovery of internal standard was 74.28% with a % coefficient of variation of of 1.4 %. Matrix effects were not observed. The method is validated as per ICH guidelines

Introduction

Celiprolol(Figure 1) is a medication in the class of beta blockers, used in the treatment of high blood pressure. It is a β_1-selective adrenoceptor antagonist (beta-blocker), which acts as a weak agonist at β_2-adrenoceptors [1]. It lowers blood pressure in hypertensive patients at rest and on exercise. The effects on heart rate and cardiac output are dependent on the pre-existing background level of sympathetic tone. Under conditions of stress such as exercise, Celiprolol attenuates chronotropic and inotropic responses to sympathetic stimulation. However, at rest minimal impairment of cardiac function is seen [2]. A recent clinical trial has suggested a use for this medication in the prevention of vascular complications of a rare inherited disease called vascular Ehlers–Danlos syndrome. This study demonstrated decreased incidence of arterial rupture or dissection (a specific type of arterial rupture in which the layers of the vessel separate prior to complete failure of the artery wall) [3-6]. Celiprolol is absorbed from the gastro intestinal tract (GIT) in a nonlinear fashion; the percentage of the dose absorbed increases with increasing dose. Occasional side effects with Celiprolol are usually mild and transient have occurred. These include headache, dizziness, fatigue, nausea, somnolence and insomnia (sleep disturbances). Additional side effects associated with β_2 agonist activity, tremor and palpitations, have been reported. These effects usually do not require withdrawal of therapy. Depression, asthmatic dyspnoea and hypersensitivity pneumonitis have been reported rarely.

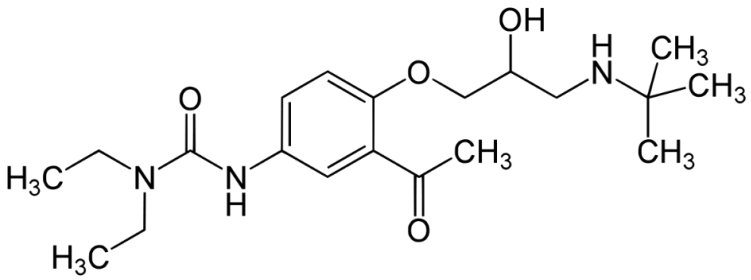

Figure 1: Chemical Structure of Celiprolol

Chemical name	:	(*RS*)-*N'*-{3-acetyl-4-[3-(*tert*-butylamino)-2-hydroxypropoxy]phenyl}-*N,N*-diethylurea
Molecular formula	:	$C_{20}H_{33}N_3O_4$
Molecular weight	:	379.49g/mol
Solubility	:	Soluble in Ethanol, Methanol and Water

Literature survey reveals that very few analytical methods have been reported for the estimation of Celiprolol using different analytical techniques in single and combined with other drugs [7- 15].**Belal et al**(7) was developed a simple, accurate, and sensitive reversed-phase HPLC method for the simultaneous determination of celiprololHCl (CE) and chlorthalidone (CT). Good chromatographic separation was achieved using a 250 mm × 4.6 mm i.d., 5µm particle size Hypurity C8 column. Mobile phase containing a mixture of methanol and 0.04 M phosphate buffer (35:65, *u/u*) at pH 7.0 was pumped at a flow rate of 1.2ml min−1 with UV detection at 225nm. Lisinoprildihydrate (LIS) was used as internal standard. The method showed good linearity in the ranges 0.2–20 and 0.2–10µg ml−1 with limits of detection 0.06 and 0.04µg mL−1 and limits of quantification 0.20 and 0.14µg mL−1 for CE and CT, respectively. The suggested method was successfully applied to the simultaneous analysis of the studied drugs in their synthetic mixture and co-formulated tablet. The method was further extended to the determination of CE in biological fluids. The proposed method was also applied to the determination of the studied drugs in presence of some coadministered or related drugs such as atenolol, propranolol, acetazolamide, enalapril, nicardipine, triamterene, and hydrochlorothiazide without any interference.

Akiko Itohda et al(8) have been developed and validated a new method of analysis for the determination of plasma celiprolol concentration. Plasma samples (1 ml) were pre-purified by solid-phase extraction with Bond Elut® C18. The separation was achieved with XBridge™ C18 column (150 mm × 3.0 mm i.d., 3.5 Î¼m) at 35 °C using a mixture of acetonitrile and 10 mMammonium acetate buffer (pH 10.5) (34:66, v/v) under isocratic conditions at a flow rate of 0.4 ml/min. The peak was detected using a fluorescence detector at excitation 250 nm and emission 482 nm. Retention times for the internal standard (acebutolol) and celiprolol were 4.2 min and 6.3 min, respectively. Calibration curves were linear over the range of 1.0–1000 ng/ml (r > 0.999), with a limit of quantification at 1.0ng/ml. Intra- and inter-assay precision (relative standard deviation) were less than 13.3% and the accuracy (relative error) was −5.1% to 11.5% at four different concentrations. This proposed method was successfully applied to a study of pharmacokinetic interactions between celiprolol and apple juice in humans. **Hefnawyet al.,**(9)has been developed a sensitive and selective high performance liquid chromatography (HPLC) method for the simultaneous determination of celiprolol enantiomers in human plasma, urine, and pharmaceutical formulation. Enantiomeric resolution was achieved on cellulose tris(3,5- dichlorophenylcarbamate) immobilized onto spherical porous silica chiral stationary phase known as Chiralpak IC with a fluorescence detection. The mobile phase consisted of n-hexane:ethanol:triethylamine (70:30:0.4%, v/v/v) has been used with a flow rate of 0.5 mL/min. S-(-)-acebutolol was used as the internal standard. The assay involved the use of acetonitrile for deproteinization of human plasma, and solid phase extraction (SPE) method for human urine samples prior to HPLC analysis. The method was validated in compliance with the international conference on harmonization (ICH) guidelines. The calibration curves were linear over the range of 5-250ng/ml for each enantiomer. The detection limits of each enantiomer were 1.5 and 2.5ng/ml in plasma and urine, respectively. The developed method applied for the determination of celiprolol enantiomers in urine, plasma and pharmaceutical formulations. Stress degradation studies as well as assay method indicated

that, the method can be used as stabilityindicating method and chiral quality control for celiprolol enantiomers by HPLC.**Verbesselt et al.,**(10) has been developed a method for the determination of total celiprolol (sum of enantiomers) or the enantiomers (R)-celiprolol and (S)-celiprolol in plasma by high-performance liquid chromatography with UV and fluorescence detection. After extraction from alkalinized plasma with methyl-tert.-butyl ether and back-extraction into 0.01 M HCl (for total celiprolol determination) or after evaporation of the organic phase and derivatisation with R(-)-1-(1-naphthyl)ethyl isocyanate (enantiomer determination), total celiprolol or its diastereomeric derivatives were chromatographed on a reversed-phase HPLC column with a mixture of acetonitrile and phosphate buffer pH 3.5 (+0.05% triethylamine). Acebutolol was used as internal standard. Linearity was obtained in the range of 5 to 2000ng/ml for total and 2.5 to 500ng/ml for enantiomer determination. Intra-day and inter-day variation was lower than 10%. The method can be applied for analysis of plasma samples obtained from patients treated with oral racemic celiprolol doses. **Ying He et al.,** (11) method was established a reversed-phase high-performance liquid chromatographic (RP-HPLC) for the determination of the enantiomers of 7 aryloxyaminopropanol drugs (atenolol, sotalol, celiprolol, carvedilol, metoprolol, propranolol and propafenone) in transport medium. The method involved liquid-liquid extraction of chiral drugs from transport medium, and employed 2, 3, 4, 6-tetra-*O*-acetyl-β-D-glucopyranosylisothiocyanate (GITC, 1.0 mg/mL in acetonitrile) as a pre-column chiral derivatization reagent. After derivatization, the products were separated on an Agilent Zorbax C8 column (150 mm×4.6 mm, 5μm) at 25 °C. The mobile phase consisted of a mixture of acetonitrile and 0.01 M phosphate buffer (pH 3.5). The present methods were specific for the determination of enantiomers of each chiral drug. The absolute recoveries of the enantiomers and internal standards were >78%. The relative recoveries of the enantiomers were approximately 100%. The intra- and inter-day variations were <15%. The method was reproducible and sufficiently sensitive to determine the enantiomers of seven aryloxyaminopropanol drugs in transport medium. The method could be used to study the transport of atenolol, sotalol, celiprolol, carvedilol, metoprolol, propranolol and propafenone.**Hartmann et al.,** (12) was described the quantitative enantiospecific determination of the beta 1-selective adrenergic antagonist (R, S)-celiprolol in human plasma and urine. It involves a two-step liquid-liquid extraction of celiprolol from biological material and separation of the underivatized enantiomers by highperformance liquid chromatography on a chiral stationary phase (cellulose tris-3, 5-dimethylphenyl carbamate, coated on silica gel) with fluorimetric detection. R-(+)-Propranolol was used as an internal standard. The detection limits of 1.5ng/ml enantiomer in plasma and 2.5ng/ml enantiomer in urine at signal-to-noise ratios higher than 3 permit the performance of pharmacokinetic studies after therapeutic doses.**Braza AJ et al.,** (13) were described two reversed-phase HPLC methods with UV detection to quantify celiprolol and oxprenolol in human plasma. The analytical methods for the determination of both drugs used the same reversed-phase HPLC column, mobile phase and extraction procedure. Linearity was obtained in the ranges 15.63-1000 and 25-800ng/ml for celiprolol and oxprenolol, respectively. Intra-day and interday variation was lower than 14%. After validation of the methods, analytical error functions were established as S.D. (ng/ml)=3.096+0.041C for celiprolol and S.D. (ng/ml) =8.906+8.075x10(-8)C3 for oxprenolol. **Z. Vujic et al.,**(14)were compared Spectrophotometric and UV-densitometry methods for the determination of celiprolol hydrochloride in tablet formulations. Celiprolol hydrochloride and benzylorange react in a 1:1 ratio in the presence of phosphate buffer, producing a yellow, chloroform-extractable ion pair with an absorption maximum at 401 nm. The conditional stability constants were determined by Sommer and Job's method of non-equimolar solutions. The repeatability of the method was tested by analyzing Selectol tablets. Spectrophotometric results were compared with those obtained UV-densitometrically on Silica Gel GF254 plates with acetone–methanol–triethylamine as mobile phase. There was no significant difference in accuracy between the two methods;however. the densitometric method was more rapid.**Rutledge et al.,** (15) has been described a simple and reproducible method for the simultaneous determination of the beta 1-selective adrenergic blocker, celiprolol, and the calcium antagonist, verapamil, in human plasma. It involves a two-step liquid-liquid extraction and separation using a C18 column with ultraviolet detection at 237nm. Deacetyldiltiazem is used as the internal standard. Within-day and between-day coefficients of variation are less than 10%. The lower limits of detection are 4, 2, and 4ng/ml for celiprolol, deacetyldiltiazem, and verapamil, respectively. The assay has clinical applicability.Based on the literature studied only five bioanalytical methods were described for analysis of Celiprolol in plasma. The present work is aimed to develop a better bioanalytical method for Celiprolol in human plasma.

Materials and Methods

Instrumentation

The author has attempted to develop a liquid chromatographic method for the determination of Celiprolol using isocratic Shimadzu HPLC equipment comprising of binary LC 10AT vp pumps, SIL 10AD vpAutosampler, CTO 10A vp column oven, and

PhenomenexC18 column (150 X 4.6 mm id, 5 µm), and SPD 10Avp UV-Visible detector. All the components of the chromatographic system were controlled using SCL-10A vp System controller. Data acquisition was done using LC Solutions version1.23 SP 1 software.

Table 1: Instruments and Equipments

HPLC System	Shimadzu
Deep Freezer	Sanyo (-86°C) VIP Series
Microbalance	Sartorius
Vibramax	Heidolph
Vacuum pump	Millipore
Refrigerator	Samsung
pH meter	Orion
Micropipettes, Multippette and Micro tips	Brand and Eppendorf
Refrigerated Centrifuge (-4^{o}C)	Heraeus
Poly propylene tubes	Torson's
Water Purification System	Elix 10 / Milli-Q gradient
Ultra Sonicator	Power Sonic510, (Hwashin Technology)
Nitrogen Evaporator	ZymarkTurbovap LV station, Caliper

Drug and Internal standard

The working standard drugCeliprololhaving99.50% w/w purity was kindly provided as gift sample by LaurusPharma Ltd., Hyderabad and the internal standard Metoprololwith 99.60% purity was obtained as gift sample by M/s Roorkee Drugs Pvt. Ltd., Uttarakhand, India.

Chemicals and solvents

The chemicals used like Diethyl ether, Methanolwere of HPLC Grade, Sodium Acetate, Glacial acetic acid were of GR gradeand were purchased from Merk chemicals private limited, Mumbai.Human EDTA plasma was obtained from Lakshmisaiclinical,Hyderabad. The linearity range of the drug was checked with the Q-Test at 95 percent confidence limits which was found to be well within the acceptable limits.

Table2: Calibration curve standards

ID	Concentration (ng/ml)
CC-01	34.02
CC-02	68.05
CC-03	259.45
CC-04	518.89
CC-05	778.34
CC-06	1334.30
CC-07	1704.93
CC-08	2001.44

Table 3: Q-test for lowest concentration

Range	1967.42 (CC 8- CC 1)
Module D1	34.03 (CC 1- CC 2)
Ratio 1	0.01783 (D1/Range)
Q 95%	Theoretical value 0.52

Table 4: Q-test for highest concentration

Range	1967.42 (CC 8- CC 1)
Module D2	296.51 (CC 8- CC 7)
Ratio Q2	0.1507 (D2/Range)
Q 95%	Theoretical value 0.52

Preparation of Solutions

Preparation of 20mM Sodium acetate buffer (pH 3.0 ± 0.05)

About 2.04 grams of Sodium acetate was weighed accurately and transferred into a 1000 ml reagent bottle and dissolved in 200mL of Milli-Q water. The above solution was sonicated for 5 min and its pH was adjusted to 4.0 ± 0.05 with glacial acetic acid solution and made up to volume with Milli-Q water. The solution was stored at room temperature and used within 3 days from the date of preparation.

Preparation of Mobile Phase

A mixture of 3:7 ratios of 20mM Sodium acetate buffer and methanol was used as mobile for the HPLC analysis. Mobile phase was prepared by accurately measured 30 parts of 20mM Sodium acetate buffer and 70 parts of methanol was mixed in a 100ml volumetric flask, the contents were mixed and degassed using ultrasonicator, then it was filtered through 0.45µ nylon membrane filter paper using vacuum filtration. The solution was stored at room temperature and used within 7 days from the date of preparation.

Diluent

An equal ratio of Methanol and water was used as diluent in the analysis. For the preparation of diluent, 50mL of methanol was transferred into a 100mL reagent bottle and 50mL of Milli-Q water was added, mixed and sonicated for 5 minutes. The solution was stored at room temperature and use within 7 days from the date of preparation.

Rinsing solution

An equal ratio of Acetonitrile and water was used as diluent in the analysis. For the preparation of rinsing solution, 50ml of acetonitrile was transferred into a 100mL reagent bottle, 50mL of Milli-Q water was added, mixed and sonicated for 5 minutes. The solution was stored at room temperature and used within 7 days from the date of preparation. This solution was used for rinsing the injection needle of the HPLC instrument.

Extraction solvent

Diethyl ether was used for performing liquid-liquid extraction of drug from plasma samples. Required quantity of analytical reagent grade diethyl ether is transferred into a reagent bottle. This solvent was stored at room temperature and used within 7 days from the date of preparation.

Stock solution of the Drug

About 14.9 mg of Celiprololwas weighed accurately and transferred into a 20 mL volumetric flask containing 2 mL of methanol. The contents were sonicated for 5 min and then the volume made up with a further quantity of methanol to get an approximate concentration of 741.28µg/ml.The stock is then stored in the refrigerator below 10^0C until further use.

Stock solution of the internal standard

About 80.0 mg of Metaprolol was weighed accurately and transferred into a 5ml volumetric flask containing 2mlof methanol. The solution was sonicated for 5 min and then the volume made up with a further quantity of the methanol to get an approximate concentration of 15.936µg/ml. Store this stock solution below 10^0C in a refrigerator.

Internal standard dilution

0.126mL of Metoprolol stock solution was transferred into a 10ml volumetric flask and the volume made up with a mixture of methanol and water (50:50 % v/v) to obtain an approximate final concentration of 200.79µg/ml. The solution was stored at room temperature and used within 8hrs for analysis for spiking plasma samples.

Calibration Curve dilutions (CC Spiking solutions)

The calibration curve dilutions were prepared from Celiprolol stock solution as per the table 5.5 in the concentration range of 0.68to40.03µg/mL using a mixture of methanol and water (50:50) as the diluent. These dilutions (CC spiking solutions) were subsequently used for spiking the screened blank plasma.

Spiked Calibration Curve Plasma Standards

The above calibration curve dilutions (CC spiking solutions) were used to spike the screened blank human plasma matrix to prepare the plasma calibration curve standards ranging from 34.02to 2001.44ng/ml as per the table 5.6. Aliquots containing 0.600ml of the above plasma calibration curve standards were taken in pre labeled polypropylene vials which were then capped tightly and stored in a freezer at -70^0C.

Table 5:Preparation of Aqueous Calibration Curve Standards

Solution ID	Concentration (µg/ml)	Volume Taken (ml)	Volume of Diluent Taken (ml)	Total Volume (ml)	Final Concentration (µg/ml)	ID of Aqueous Solution Prepared
CEL-ST	741.28	0.270	4.730	5.000	40.03	AQ-CC-08
CEL-ST	741.28	0.230	4.770	5.000	34.10	AQ-CC-07
CEL-ST	741.28	0.180	4.820	5.000	26.69	AQ-CC-06
CEL-ST	741.28	0.105	4.895	5.000	15.57	AQ-CC-05
CEL-ST	741.28	0.070	4.930	5.000	10.38	AQ-CC-04
CEL-ST	741.28	0.035	4.965	5.000	5.19	AQ-CC-03
CEL-INT	75.61	0.090	4.910	5.000	1.36	AQ-CC-02
CEL-INT	75.61	0.045	4.955	5.000	0.68	AQ-CC-01

Table6:Preparation of Plasma Spiked Calibration Standards

Solution ID	Concentration (µg/ml)	Volume Taken (ml)	Volume of Plasma Taken (ml)	Total Volume (ml)	Final Concentration of Plasma samples (ng/ml)	ID of Plasma Spiked Samples
AQ-CC-08	40.03	0.500	9.500	10.000	2001.44	CC-08
AQ-CC-07	34.10	0.500	9.500	10.000	1704.93	CC-07
AQ-CC-06	26.69	0.500	9.500	10.000	1334.30	CC-06
AQ-CC-05	15.57	0.500	9.500	10.000	778.34	CC-05
AQ-CC-04	10.38	0.500	9.500	10.000	518.89	CC-04
AQ-CC-03	5.19	0.500	9.500	10.000	259.45	CC-03
AQ-CC-02	1.36	0.500	9.500	10.000	68.05	CC-02
AQ-CC-01	0.68	0.500	9.500	10.000	34.02	CC-01

Quality control (QC)spiking solutions

QC spiking solutions from Celiprololstock solution were prepared as per the table 5.7in the concentration range from 0.71 to 37.06µg/ml using a mixture of methanol and water (50:50) as the diluent. These QC spiking solutions were subsequently used for spiking the screened blank plasma.

Spiked QC Plasma Sample solutions

The above QC spiking solutions were used to spike the screened blank human plasma to prepare the plasma quality control plasma samples ranging from 35.54to 1853.19ng/ml as per the table given 5.8. Aliquots containing 0.600 mL of the above plasma calibration curve standards were taken in pre labeled polypropylene vials which were then capped tightly and stored in a freezer at -70^0C.

Table 7:Preparation of Aqueous Quality Control Samples

Solution ID	Concentration (µg/ml)	Volume Taken (ml)	Volume of Diluent Taken (ml)	Total Volume (ml)	Final Concentration (ng/ml)	ID of Aqueous Solution Prepared
CEL-ST	741.28	0.250	4.750	5.000	37.06	AQ-HQC
CEL-ST	741.28	0.135	4.865	5.000	20.01	AQ-MQC
CEL-INT	75.61	0.140	4.860	5.000	2.12	AQ-LQC
CEL-INT	75.61	0.047	4.953	5.000	0.71	AQ-LLOQ

Table 8: Preparation of Plasma spiked Quality Control Samples

Solution ID	Concentration (µg/ml)	Volume Taken (ml)	Volume of Diluent Taken (ml)	Total Volume (ml)	Final Concentration of Plasma samples (ng/ml)	ID of Spiked Solution Prepared
AQ-HQC	37.06	0.500	9.500	10.000	1853.19	HQC
AQ-MQC	20.01	0.500	9.500	10.000	1000.72	MQC
AQ-LQC	2.12	0.500	9.500	10.000	105.85	LQC
AQ-LLOQ	0.71	0.500	9.500	10.000	35.54	LLOQ

METHOD DEVELOPMENT

For optimum detection and quantitation of Celiprolol in human plasma by liquid chromatography, it was necessary to maintain the chromatographic condition throughout the experimentation. For developing a new simple suitable analytical method for the estimation of Celiprolol in human plasma, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all the other conditions constant. The following studies were conducted for this purpose. A non-polar Phenomenex ODS-2, C_{18}column (150 X 4.6 mm id) was chosen as the stationary phase for this study.

The mobile phase and the flow rate

A number of trials were made to find out the ideal solvent system (mobile phase) for eluting the drugCeliprolol and its internal standard Metoprolol. To effect ideal separation of the drug under isocratic conditions, mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases on a C_{18} stationary phase. Preliminary trails confirm that methanol was the better solvent for elution of the standard drug and internal standard with high resolution.

The mobile phase containing Methanol as high percentage and water, Acetonitrile with minimum usage doesn't improve the separation of the standard drug. Hence different ratios of Methanol with different buffer solutions were tried. Better peak resolution with less tailing and high theoretical plates was observed with the 30:70 (v/v) ratio of 20mM Sodium acetate buffer (pH 4.0 ± 0.05) and methanol. Hence this binary solution was used as mobile phase for the separation and estimation of Celiprolol. Flow rate of the mobile phasewas tested from 0.8ml/min to 1.2ml/min in order to get sharp symmetric peaks with high resolution, minimum usage of the solvents and low pump pressure. A mobile phase flow rate of 1.0mL/min was found to be suitable in the present study.

Detection wave length

The sensitivity of the HPLC Method depends upon the proper selection of the detection wavelength. An ideal wavelength is one thatgive good response to the detector. The UV absorption spectrum of working standard solution of the Celiprolol was recorded separately on UV-Visible spectrophotometer and

found the maximum absorbance wavelength at 210nm.Hence the response of the detector was recorded continuously at maximum wavelength 210nm.

Retention time of drug and internal standard

In the optimized conditions, Celiprolol and Metoprolol separately with high resolution and obey the system suitable criteria. Prominent fixed retention time of 4.08min for Celiprolol and 5.38min for Metoprolol was observed. The typical standard chromatogram was shown in figure 4

Data acquisition and processing

The chromatograms were obtained and data were processed by the peak area ratio method using the LC solution software. The concentrations of the unknown samples were calculated from the following equation of the regression analysis of the spiked plasma calibration graph using $1/X^2$ as weighting factor.

$$Y = m X + C$$

X = Concentration of Analyte / Concentration of Internal standard
Y = Area of Analyte / Area of Internal standard (area ratio)
m = Slope of the calibration curve
C = Y- intercept value

Table 9: Optimized Chromatographic Conditions

Parameter	Specifications
Column	Phenomenex ODS-2, $C_{18,}$ (4.6 X 150 mm, 5µ)
Mobile phase	20mMSodium acetate buffer (pH 4.0) and Methanol(30: 70 v/v)
Flow rate	1.0ml/min
Run time	7.0 min
Column oven temperature	Ambient
Auto sampler temperature	4^0C
Volume of injection	20µl
Detection wave length	210 nm
Retention time of Celiprolol	4.08min.
Retention time of Metoprolol	5.38min.

Extraction process of plasma samples

Step 1: 400 micro liters of the spiked plasma calibration curve standards and the quality control samples were transferred to a 2.0 ml eppendorf micro centrifuge tubes.
Step2: To this 50 µL of Metoprolol dilution (internal standard;approximately100µg/ml) was added and vortexed for ten seconds.
Step 3: 1.0 ml of diethyl ether is then added and mixed.
Step 4: The samples are then subjected to flash-freezing using a mixture of dry-ice and acetone.
Step 5: The supernatant is then transferred into another labeled polypropylene tubes and evaporated to dryness under nitrogen at 40°C.
Step 6: The dried residue is reconstituted with 0.3 ml of mobile phase, vortexedthoroughly and transferred into autosampler vials for analysis. An injection volume of 20µL is taken during final analysis.

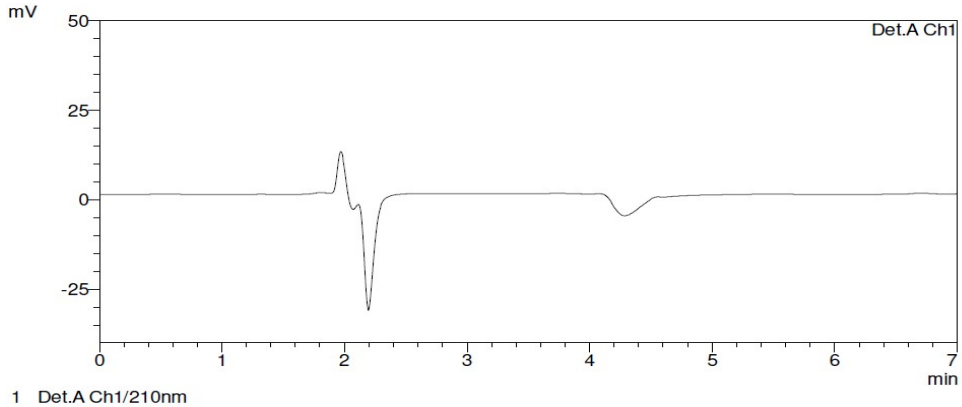

Figure 2: Chromatogram of the extracted blank plasma sample

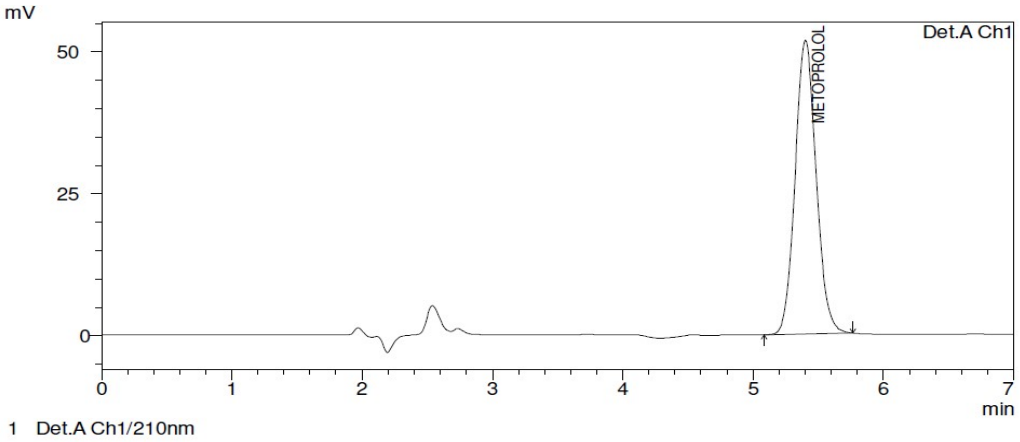

Figure3: Chromatogram of Metoprolol spiked blank plasma sample

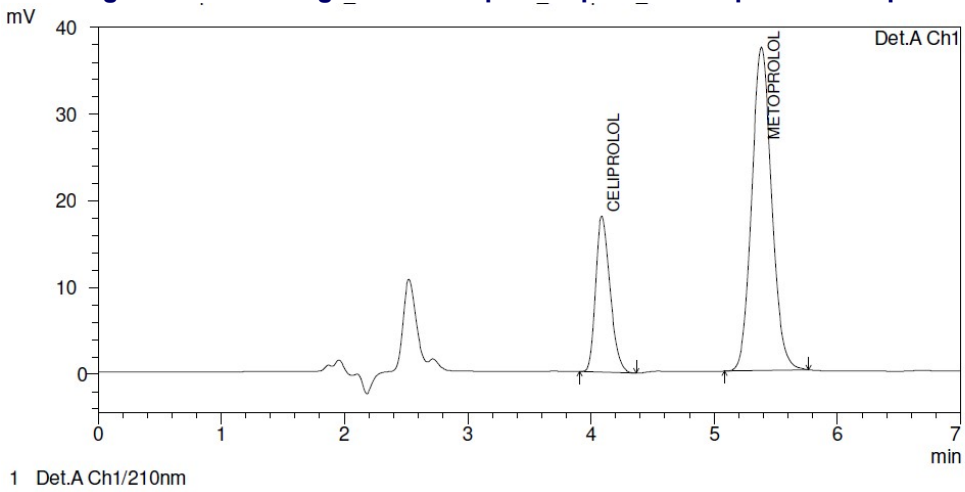

Figure 4: Chromatogram of Celiprolol (drug) and Metoprolol (IS) extracted from human plasma

METHOD VALIDATION

Carryover test

This was determined by sequentially injecting the extracted blank plasma sample, the extracted ULOQ standard, once again the extracted blank plasma sample and calculating the percentage of the residual analyte and the internal standard carried over to the latter blank plasma sample.

Table10: % carryover of the drug and the internal standard

Sample Name	Area at the RT of Celiprolol	% Carryover of Celiprolol	Area at the RT of Metoprolol (ISTD)	% Carryover of ISTD
Mobile phase/Reconstitution Solution-I	0	0.0	0	0.0
Highest Aqueous standard (AQS ULOQ)	926646		884565	
Extracted blank-I	0	0.0	0	0.0
Highest Extracted standard (Ext ULOQ)	594313		534556	

Screening of plasma lots and specificity

Accordingly, specificity of the HPLC method was evaluated by its successful application to determine drugs in their samples with high recovery and without any interference. The specificityof the present method was evaluated by checking the blank EDTA (Ethylene di-amine tetra acetic acid) plasma (without spiking with Celiprolol) obtained from different blood donors. Six different lots of blank plasma were screened and all of them were found to have no significant endogenous interferences at the retention times of the analyte and the internal standard. The same human EDTA plasma lots free of interfering substances were used to prepare the calibration curve standards and the quality control samples for the validation study.Retention time of the peak in the chromatogram of standard and spiked human plasma was same without interference from excipients, additives or biological fluid components. Chromatograms of blank and standard were shown in figure 2 and.4 respectively.

Table 11: % interferences at the retention times of the drug and the internal standard

Human Plasma Id	Drug			Internal Standard		
	Response in Blank	Response in LLOQ	% Interference	Response in Blank	Response in LLOQ	% Interference
1	216	4218	5.12	1568	524567	0.30
2	188	4786	3.93	1204	523833	0.23
3	215	4645	4.63	1558	525666	0.30
4	176	4985	3.53	2106	527506	0.40
5	104	5124	2.03	2206	529352	0.42
6	98	5228	1.87	2015	531205	0.38
Average	166.2	4831	3.519	1776.2	527021.4	0.337
Total Number of Matrices	**6**	**Number of matrices meeting the requirements**				**6**
Percentage of Matrices meeting the selectivity criteria						**100**

Linearity

Calibration curve representing the relation between the concentrations of drug versus the ratio of the peak area of standard drug to internal standard were constructed.The linearity of the method was determined by a weighted ($1/X^2$, where, X is concentration) least square regression analysis of the standard plots associated with the eight-point standard curve for Celiprolol. The calibration curve was linear in the range of 34.02to 2001.44ng/ml of the drug as shown in Fig 5.5. A straight-line fit made through the data points by least square regression analysis showed a constant proportionality with minimal data scattering. The regression coefficient (r^2) ranged from 0.9943 to 0.9975 for Celiprolol. Results indicate high sensitivity of the proposed method. According to ICH recommendation, the approach based on the S.D. of the response and the slope was used for determining the detection and quantitation limits. Linearity results were shown in table12 and calibration curve was shown in figure 5.

Table 12: Back calculated concentrations of Celiprolol and calibration curve parameters

Summary of the Calibration Curve Parameters- 3 Curves									
Batch Id	**CC ID**	**CC1**	**CC2**	**CC3**	**CC4**	**CC5**	**CC6**	**CC7**	CC8
	Nominal Concentration (ng/ml)	34.02	68.05	259.45	518.89	778.34	1334.30	1704.93	2001.44
PA 01	-	34.83	66.27	265.27	516.19	773.91	1345.68	1615.18	2046.21
PA 02	-	36.42	62.72	251.07	530.39	788.91	1359.66	1575.76	2073.83
PA 03	-	31.60	68.52	256.70	547.72	788.39	1338.83	1628.92	2086.43
N		3	3	3	3	3	3	3	3
Mean		34.282	65.835	257.678	531.434	783.737	1348.055	1606.619	2068.824
SD		2.4542	2.9227	7.1509	15.7924	8.5185	10.6143	27.5958	20.5745
% CV		7.16	4.44	2.78	2.97	1.09	0.79	1.72	0.99
% Accuracy		100.76	96.75	99.32	102.42	100.69	101.03	94.23	103.37

Table 13: Results of regression analysis of the linearity data

Batch Id	CC ID	Y-Intercept	Slope	Regression Coefficient (r^2)
PA 01	**1**	-0.00024	0.00030	0.9975
PA 02	**2**	0.00041	0.00035	0.9943
PA 03	**3**	0.00410	0.00034	0.9967
	N	**3**	**3**	**3**
	Mean	**0.001423333**	**0.00033**	**0.99617**

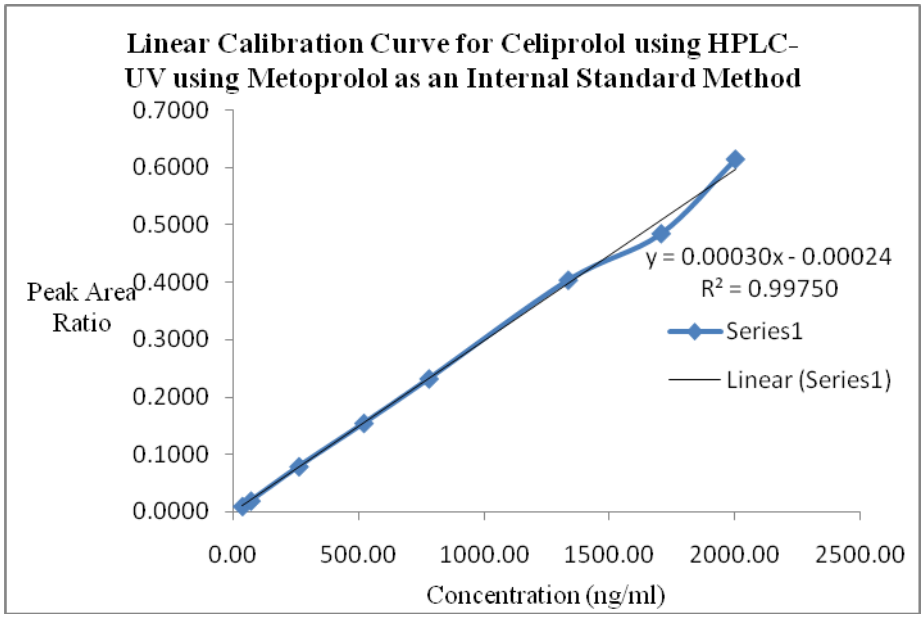

Fig. 5: Calibration curve for Celiprolol (P & A - 01)

Precision

The precision of the method was determined by intraday precision and interday precision. The intraday precision was evaluated by analysis of plasma samples containing Celiprolol at three different concentrations containing internal standard using nine replicate determinations for three occasions. The interday precision was similarly evaluated over two-week period. Precision studies were carried out for three levels at nine times and three occasions. The mean concentration, standard deviation and % CV were calculated. The calculated relative standard deviation values were found to be small indicating good repeatability and reliability of the proposed method. The results and their statistical analysis were summarized in Table 14.

Table 14: Precision and Accuracy of QC samples for Celiprolol (within-batch)

P & A ID		Observed Concentration (ng/ml)			
	QC ID	HQC	MQC	LQC	LLOQ QC
PA-01	1	1782.28	1074.09	109.90	35.41
	2	1826.58	996.68	104.18	37.80
	3	1905.35	972.36	109.65	37.36
	4	1824.21	1023.56	101.22	35.41
	5	1811.34	1088.97	102.34	33.67
	6	1798.65	1062.21	108.76	36.41
N		**6**	**6**	**6**	**6**
Average		**1824.73**	**1036.31**	**106.01**	**36.01**
Standard Deviation		**42.812**	**46.248**	**3.891**	**1.509**
CV (Precision %)		**2.35**	**4.46**	**3.67**	**4.19**
Nominal Concentration		**1853.19**	**1000.72**	**105.85**	**35.54**
Accuracy (%)		**98.46**	**103.56**	**100.14**	**101.33**
PA02	13	1812.58	1092.35	111.77	36.01
	14	1868.59	1019.60	106.57	38.67
	15	1945.36	992.78	111.95	38.15
	16	1821.29	1021.92	101.06	35.35
	17	1792.32	1077.54	101.27	33.32
	18	1766.45	1043.20	106.81	35.76
N		**6**	**6**	**6**	**6**
Average		**1834.433**	**1041.231**	**106.571**	**36.210**
Standard Deviation		**64.057**	**37.746**	**4.786**	**1.957**
CV (Precision %)		**3.49**	**3.63**	**4.49**	**5.41**
Nominal Concentration		**1853.19**	**1000.72**	**105.85**	**35.54**
Accuracy (%)		**98.99**	**104.05**	**100.68**	**101.89**
PA 03	19	1786.48	1076.62	110.16	35.50
	20	1860.00	1014.91	106.08	38.49
	21	1918.90	979.28	110.43	37.63
	22	1812.37	1016.91	100.56	35.18
	23	1786.41	1073.98	100.93	33.21
	24	1762.74	1041.01	106.59	35.68
N		**6**	**6**	**6**	**6**
Average		**1821.149**	**1033.785**	**105.792**	**35.948**
Standard Deviation		**58.245**	**37.722**	**4.295**	**1.880**
CV (Precision %)		**3.20**	**3.65**	**4.06**	**5.23**
Nominal Concentration		**1853.19**	**1000.72**	**105.85**	**35.54**
Accuracy (%)		**98.27**	**103.30**	**99.94**	**101.16**

Table 15: Global Precision and Accuracy results of QC samples for Celiprolol(between batches)

QC ID	HQC	MQC	LQC	LLOQ QC
N	18	18	18	18
Average	1826.772	1037.109	106.123	36.056
Standard Deviation	6.872	3.787	0.402	0.137
CV (Precision %)	0.38	0.37	0.38	0.38
Nominal Concentration (ng/ml)	1853.19	1000.72	105.85	35.54
Accuracy (%)	98.57	103.64	100.25	101.46

Recovery

The accuracy of the drug was calculated by comparing the concentration obtained from the relative recovery of drug supplemented plasma to the actually added concentration. To drug supplemented plasma, standard Celiprolol solution and internal standard solution were added. The resulting sample solution was analysed and the response factor was calculated. The absolute recovery of Celiprolol was determined by comparing the response factor of the drug obtained from the plasma with response factor obtained by the direct injection of Celiprolol in mobile phase at three different levels. Recovery studies were carried out for three levels at six times and the % recovery, mean, standard deviation and % CV was calculated. Mean Recovery for Celiprololranges from 51.2% to 55.4% (Mean Recovery: 52.84%) and themean recovery obtained for Metoprololranged from 58.99% to 62.71 %.(Mean Recovery: 60.36 %). Recovery results were shown in table 5.16 for standard drug and 5.17 for internal standard.

Table 16: Recovery of Celiprololin human plasma

S. No.	EXT HQC	AQS HQC	% Recovery	EXT MQC	AQS MQC	% Recovery	EXT LQC	AQS LQC	% Recovery
1	516711	928246	55.67	236117	418558	56.41	21136	42056	50.26
2	516139	913777	56.48	214060	417044	51.33	21874	42536	51.42
3	514313	945883	54.37	213124	447874	47.59	19653	40345	48.71
4	523945	942170	55.61	239659	456814	52.46	21432	37913	56.53
5	505300	903725	55.91	211705	408286	51.85	21415	38389	55.78
6	513542	944464	54.37	212804	447202	47.59	19624	40284	48.71
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Average	**514991.6**	**929710.8**	**55.4**	**221244.9**	**432629.7**	**51.2**	**20855.5**	**40254.0**	**51.9**
SD	**6015.38**	**17695.63**	**0.86**	**12962.01**	**20313.42**	**3.33**	**972.08**	**1866.61**	**3.46**
% CV	**1.17**	**1.90**	**1.54**	**5.86**	**4.70**	**6.50**	**4.66**	**4.64**	**6.66**
Mean Recovery	**52.84**								
SD	**2.250**								
% CV	**4.26**								

Table 17:Recovery of Metoprolol (Internal standard)

S. No.	Ext HQC	Aqs HQC	% Recovery	Ext MQC	Aqs MQC	% Recovery	Ext LQC	Aqs LQC	% Recovery
1	518398	882563	58.74	530286	839821	63.14	537174	856864	62.69
2	531240	885012	60.03	512958	870383	58.93	513673	875038	58.70
3	522275	892271	58.53	504240	879189	57.35	522445	870345	60.03
4	525656	895801	58.68	538240	916581	58.72	544694	772463	70.51
5	520084	875277	59.42	507315	852105	59.54	502886	789722	63.68
6	521492	890933	58.53	503484	860286	58.53	521661	860336	60.63
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Average	**523190.7**	**886976.2**	**58.99**	**516087.2**	**869727.5**	**59.37**	**523755.6**	**837461.3**	**62.71**
SD	**4630.64**	**7507.13**	**0.61**	**14685.41**	**26755.38**	**1.98**	**15243.34**	**44490.26**	**4.23**
% CV	**0.89**	**0.85**	**1.03**	**2.85**	**3.08**	**3.34**	**2.91**	**5.31**	**6.74**
Mean Recovery					**60.36**				
SD					**2.047**				
% CV					**3.39**				

Stability of the drugs in stock solution

Short-term stability of the drugs in stock solution

Short term stock solution stability for Celiprololand internal standard were performed at the stock solution concentration by using six consecutive injections of aqueous standard equivalent to mg/ml concentration and working concentration respectively after storage of at least 9hours at ambient temperature. Stability was assessed by comparing the stock dilutions of Celiprolol and Internal Standard prepared from the freshly prepared stock solutions (comparison) against stock dilutions of Internal Standard prepared from the stock solutions stored at ambient temperature (stability). Short term stock solution stability was evaluated by comparing the mean response of stability samples against mean response of comparison samples. The percent stabilities were found to be 100.44 % and 103.58 % for Celiprolol and Metoprolol respectively. Results of the short term stability were shown in Table 18.

Table 18: Short-term stability of Celiprolol and Metoprolol

	Drug		Internal Standard	
Injection No.	**AQS MQC Response**		**AQS MQC Response**	
	Fresh Stock	**Room Temp Stock**	**Fresh Stock**	**Room Temp Stock**
1	4118847	4343029	814480	839471
2	4306866	4448822	854202	871161
3	4278486	4484124	842281	867267
4	4172991	4407018	820960	861525
5	4834034	4576093	860434	885064
6	4816978	4297260	858756	843432
N	**6**	**6**	**6**	**6**
Average	**4421367.0**	**4426057.7**	**841852.2**	**861320.0**
SD	**320479.82**	**100245.32**	**19846.63**	**17282.31**
% CV	**7.25**	**2.26**	**2.36**	**2.01**
% Stability	**100.44**		**103.58**	
% Change (100 - % Stability)	**-0.44**		**-3.58**	
Stock Concentration µg/ml	**75.61**	**75.36**	**200.79**	**198.33**
Correction Cc	**1.0034**		**1.0124**	

Long-term stability of drugs in stock solution

Long term stock solution stability for Celiprololand internal standard were performed at the stock concentration by using six consecutive injections of aqueous standard equivalent to mg/ml concentration and working concentration respectively after storage of at least 11 days or for any other suitable period by considering the expected duration of the usage of the stock solution in the refrigerator at 10°C. Stability was assessed by comparing the stock dilutions of Celiprolol and Internal Standard prepared fromthe freshly prepared stock solutions (comparison) against stock dilutions of Celiprololand internal standard prepared from the stock solutions stored at 10°C (stability). Long termstock solution stability was evaluated by comparing the mean response of stability samples against mean response ratios of comparison samples.The percent stabilities obtained were 96.22% and 103.48% for CeliprololandMetoprolol respectively. Results of the long term stability were shown in Table 19.

Table 19:Long-term stability of Celiprolol and Metoprolol

	Drug		Internal Standard	
Injection No.	**AQS STD H Response**		**AQS STD H Response**	
	Fresh Stock	**Refrigerator Stock**	**Fresh Stock**	**Refrigerator Stock**
1	4118847	4125878	814480	849545
2	4306866	4213034	854202	849382
3	4278486	4283621	842281	852523
4	4172991	4429658	820960	853771
5	4834034	4172639	860434	894800
6	4816978	4215612	858756	862831
N	**6**	**6**	**6**	**6**
Average	**4421367.0**	**4240073.8**	**841852.2**	**860475.3**
SD	**320479.82**	**106542.25**	**19846.63**	**17518.16**
% CV	**7.25**	**2.51**	**2.36**	**2.04**
% Stability	**96.22**		**103.48**	
% Change (100 - % Stability)	**3.78**		**-3.48**	
Stock Concentration µg/ml	**75.61**	**75.36**	**200.79**	**198.33**
Correction Factor	**1.0034**		**1.0124**	

Stability of drugs in biological matrix

Freeze-thaw stability

Freeze thaw(FT) stability of the spiked quality control samples were determined after three freeze thaw cycles stored at -20±5°C. Six replicates of each HQC and LQC samples were used for assessing each freeze thaw experiment (for first and fifth cycle at both the freezing temperatures). The first freeze-thaw cycle was of at least 24hours followed by minimum of 12 hours for subsequent cycles. Freeze thaw stability samples were processed and analyzed along with freshly spiked calibration curve and comparison samplesin screened biological matrix. Freeze thaw stability on the basis of % change of the samples was evaluated. Results of the long Freeze thaw stability were shown in Table 5.20 and 5.21. The Freeze-thaw stability values for the calibration curve standards of Celiprolol in plasma after 3 FT cycles were 93.28% and 105.98% at low and high concentrations respectively.

Table 20:Back calculated concentrations for CC Standards of Celiprolol (3 cycles)

Batch ID	CC ID	CC A	CC B	CC C	CC D	CC E	CC F	CC G	CC H
	Nominal Concentration	34.02	68.05	259.45	518.89	778.34	1334.30	1704.93	2001.44
PA 01	-	36.06	63.97	253.42	535.32	796.23	1372.24	1590.33	2093.00
% Accuracy		105.98	94.01	97.68	103.17	102.30	102.84	93.28	104.57

Table 21:Freeze-thaw stability of Celiprolol in plasma (3 cycles)

Freeze-Thaw Stability (3 Cycles)	Fresh HQC	Stability HQC	Fresh LQC	Stability LQC
Aliquot ID	Obtained concentration (ng/ml)			
	Fresh HQC	Stability HQC	Fresh LQC	Stability LQC
1	1876.55	1788.56	108.88	109.95
2	1881.98	1795.60	106.78	105.22
3	1925.21	1888.11	105.41	109.76
4	1990.44	1878.98	106.32	108.66
5	1912.34	1834.45	108.12	106.87
6	1934.88	1925.31	107.88	109.88
N	6	6	6	6
Average	1920.233	1851.835	107.232	108.390
SD	41.48	54.63	1.29	1.95
% CV	2.16	2.95	1.20	1.80
Nominal Concentration	1846.97	1853.19	105.50	100.85
Mean Accuracy %	103.97	99.93	101.64	107.48
Correction Factor	0.9966		1.0461	
% Stability	96.11		105.74	

Bench top stability

For determining the bench top stability of Celiprolol in human plasma six replicates of unprocessed stability samples, which were maintained at a temperature of ~ 25^0C for 8.00 hours and six replicates of the freshly prepared quality control samples (Comparison samples) at low and high QC concentration levels (known from a freshly prepared calibration curve) were analyzed and the percent stability at low and high QC concentration levels was calculated by comparing the mean of the concentrations of the stability samples with that of the comparison samples. The Bench top stability values of Celiprolol in plasma after 8.00 hours were 96.98% and 106.46% at low and high concentrations respectively.

Table 22: Bench top stability of Celiprolol in Plasma(8.00 hours)

Aliquot ID	Obtained concentration (ng/ml)			
	Fresh HQC	Stability HQC	Fresh LQC	Stability LQC
1	1876.55	1806.45	108.88	109.62
2	1881.98	1772.26	106.78	103.85
3	1925.21	1916.43	105.41	112.28
4	1990.44	1909.04	106.32	112.35
5	1912.34	1847.29	108.12	109.54
6	1934.88	1959.97	107.88	107.13
N	6	6	6	6
Average	1920.233	1868.572	107.232	109.131
SD	41.48	71.93	1.29	3.24
% CV	4664.83	2724.78	8383.18	3303.01
Nominal Concentration	1846.97	1853.19	105.50	100.85
Accuracy	103.97	100.83	101.64	108.21
Correction Factor	0.9966		1.0461	
% Stability	96.98		106.46	

In-injector stability or Auto-sampler stability

Auto-sampler re-injection reproducibility was evaluated by re-injecting accepted precision and accuracy batch, which were stored preferably in either autosampler or in refrigerator for at least 24 hours or as per requirement. The In-injector stability values of Celiprololin human plasma after 48.00 hours were 97.87% and 107.24% at low and high concentrations respectively.

Table 23: In-injector stability of Celiprolol(48.00 hours)

Aliquot ID	Obtained concentration (ng/ml)			
	Fresh HQC	Stability HQC	Fresh LQC	Stability LQC
1	1876.55	1824.51	108.88	109.29
2	1881.98	1749.22	106.78	102.50
3	1925.21	1945.18	105.41	114.87
4	1990.44	1939.59	106.32	116.17
5	1912.34	1860.22	108.12	112.28
6	1934.88	1995.24	107.88	104.45
N	**6**	**6**	**6**	**6**
Average	**1920.233**	**1885.660**	**107.232**	**109.928**
SD	**41.48**	**91.06**	**1.29**	**5.56**
% CV	**2.16**	**4.83**	**1.20**	**5.06**
Nominal Concentration	**1846.97**	**1853.19**	**105.50**	**100.85**
Accuracy	**103.97**	**101.75**	**101.64**	**109.00**
Correction Factor	**0.9966**		**1.0461**	
% Stability	**97.87**		**107.24**	

Long-term stability in plasma matrix

For finding out the long-term stability of Celiprololin human plasma the stability samples were stored at a temperature of -70ºC for 15 days. Then they were analyzed along with six replicates of the freshly prepared quality control samples (Comparison samples) at low and high concentration levels. The low and high concentration levels were read from the calibration curve. The quality control samples and the calibration curve standards were prepared by spiking the freshly prepared drug dilutions in screened blank plasma. The percent stabilities at low and high QC concentration levels were calculated by using the mean of the concentrations of the stability samples and the mean of the comparison samples. The long-term stability values obtained for Celiprololin human EDTA plasma at a temperature of -70°C for 20 days were 98.78% and 107.84% at high and low concentrations respectively.

Table 24:Long-term stability of Celiprolol(15 days) at -70^{0}C in EDTA

Aliquot ID	Obtained concentration (ng/ml)			
	Fresh HQC	Stability HQC	Fresh LQC	Stability LQC
1	1876.55	1842.76	108.88	108.96
2	1881.98	1726.48	106.78	104.35
3	1925.21	1974.36	105.41	113.49
4	1990.44	1970.62	106.32	118.50
5	1912.34	1873.24	108.12	111.49
6	1934.88	2031.16	107.88	106.44
N	**6**	**6**	**6**	**6**
Average	**1920.233**	**1903.102**	**107.232**	**110.538**
SD	**41.48**	**111.14**	**1.29**	**5.11**
% CV	**2.16**	**5.84**	**1.20**	**4.62**
Nominal Concentration	**1846.97**	**1853.19**	**105.50**	**100.85**
Accuracy	**103.97**	**102.69**	**101.64**	**109.61**
Correction Factor	**0.9966**		**1.0461**	
% Stability	**98.78**		**107.84**	

Dilution Integrity

A quality control pool (containing 4002.88ng/ml of Celiprolol) was prepared in EDTA-plasma at a concentration of approximately twice the high CC standard (ULOQ) to assess the dilution integrity. The precision and accuracy for dilution integrity at 50% dilution and 25% dilution of the QC pool sample with screened blank human plasma were determined by using freshly spiked (FS) calibration curve standards. The precision for dilution integrity of Celiprololwas1.27% at 25 percent dilution and 2.34% at 50 percent dilution. The accuracy for dilution integrity of Celiprololwas102.23% for 25 percent dilution and 102.73% for50 percent dilution. Results are shown in the table 25.

Table25: Back calculated concentrations for quality control dilution samples in plasma

Dilution Integrity		
Injection No.	Obtained concentration (ng/ml)	
	25% Dilution	50% Dilution
1	4016.85	4010.82
2	4056.88	4034.57
3	4098.45	4044.35
4	4089.76	4138.84
5	4165.78	4240.76
6	4124.46	4202.82
N	6	6
Average	4092.03	4112.03
SD	51.82	96.29
% CV	1.27	2.34
Nominal Concentration	4002.88	
Accuracy	102.23	102.73

Discussion

A rapid, sensitive and rugged solid-phase extraction high performance liquid chromatography method was developed for determination of Celiprolol in human plasma. Optimization of chromatographic conditions were intended to take into account the various goals of the method development and to weigh each goal (resolutions, run time, sensitivity, peak symmetry, etc) accurately, according to the requirements can be used for the estimation of Celiprolol in plasma samples. The optimized conditions obtained a mobile phase of 20mMSodium acetate buffer (pH 4.0) and Methanol in the ratio of 30: 70 (v/v), at a flow rate of 1ml/min on Phenomenex ODS-2, $C_{18,}$ (4.6 X 150 mm, 5µ) column, and UV detection at 210nm. In this condition, retention time was found to be 4.08min for Celiprololand 5.38min for Metoprolol. The optimized conditions for estimation provided a well-defined separation between the drug, internal standard and endogenous components. The blank plasma samples showed no interference at retention time of the drug and internal standard. The method was validated for selectivity, sensitivity, linearity, precision, accuracy and stability. The selectivity of the method was evaluated by comparing the chromatograms obtained from the samples containing Celiprolol and the internal standard with those obtained from blank samples. Sensitivity was determined in terms of LLOQ (lower limit of quantification) where the response of LLOQ was at least five times greater than the response of interference in blank matrix at the retention time or mass transitions of the analyte. The optimized method for the estimation of the drug was precise as it showed < 10 % coefficient of variation at all concentrations. The blank plasma samples obtained from volunteers were analyzed and the chromatograms were recorded. Endogenous interferences were not detected at the retention time of selected drug and internal standard. The peak purity test method using UV detector was employed for selectivity studies. Some additional peaks were also observed in the sample chromatograms. These peaks, however, did not interfere with the drug and internal standard peaks. These observations showed that the developed assay method is specific and selective.

For linearity, different concentrations of standard solutions were prepared to contain 34.02µg/ml to 2001.44 ng/ml of Celiprolol containing 200µg/ml of Metoprolol. These solutions were analyzed and the peak areas and response factors were calculated. The calibration curve was plotted using response factor vs. concentration of the standard solutions. Standard curve was determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighing and statistical tests for goodness of fit. The regression coefficient (r^2) ranged from 0.9943 to 0.9975 for Celiprolol. No significant changes in the chromatographic parameters were observed when changing the experimental conditions (Analysts, instruments, source of reagents and column of similar type) and optimized

conditions (pH, mobile phase ratio and flow rate). System suitability parameters such as column efficiency (theoretical plates), resolution factor and peak asymmetry factor of the optimized methods wasfound satisfactory. The high percentage of recovery of Celiprolol was found, indicates that the proposed method is highly accurate. The percentage accuracy and precision studies obtained were less than 15% for QC sample and less than 20% for LOQ QC samples revealed that developed method was accurate and precise as per the FDA guidelines. The limit of detection (LOD) value was found to be very less. This observation showed that the developed method has adequate sensitivity. These values, however, may be affected by the separation conditions (e.g., column, reagents and instrumentation), instrumental changes (e.g., pumping systems and detectors) and use of non- HPLC grade solvents and may result in changes in signal to noise ratios. The stability of the drug spiked human plasma samples at three levels were studied for three freeze thaw cycles. The mean concentrations of the stability samples were compared to the theoretical concentrations. Similarly, short term (8 hours), long term (11 days) and standard solution stability were evaluated. The stability of the internal standards was also performed. The results showed that the selected drug was stable in plasma for about 11days when stored at frozen state. Results indicatesthat the developed method was sensitive and precise. Hence, this developed assay method can be applied for estimation of Celiprolol in different bioavailability and bioequivalence studies. The summary results were shown in table.26

Table 26: Summary of Results

Parameters	Results
Screening of plasma lots and specificity	No significant interfering peaks were observed at the retention time of analyte (Celiprolol) and Internal standard (IS; Metoprolol).
Calibration Curve	**Calibration range:** 34.02 to 2001.44ng/ml **Mean Accuracy(**%nominal**):** 94.23 – 103.37 % **Precision(**%CV**):** 0.79 – 7.16 % **Regression coefficient (r^2):** 0.9943 - 0.9975
Precision (%CV)	**Within-batch** LLOQ QC: 4.19 %LQC, MQC & HQC: 2.35 – 4.46 % **Global** LLOQ QC: 0.38 %LQC, MQC & HQC: 0.37 – 0.38 %
Accuracy (% Nominal Conc.)	**Within-batch** LLOQ QC: 101.3 %LQC, MQC & HQC: 98.46 – 103.56 % **Global** LLOQ QC: 101.46 %LQC, MQC & HQC: 98.57 – 103.64 %
Recovery	**Celiprolol** Mean % Recovery = 52.84 % ; % CV=4.26 **Metoprolol (IS)**Mean% Recovery = 60.36%, % CV = 3.39
Short term stock solution stability (9.0hrs)	**% stability of Celiprolol:** 100.44% **Metoprolol (IS) Percent stability:** 103.58%
Long term stock solution stability (11 Days)	**% stability of Celiprolol:** 96.22% **Metoprolol (IS) Percent stability:** 103.48%
Freeze – Thaw stability (3 Cycles)	**% stability:** At LQC Level: 105.74 % At HQC Level: 96.11 %
Bench top stability (8.00 hours)	**% stability:** At LQC Level: 106.46 % At HQC Level: 96.98 %
In-injector stability (48.00 hours)	**% stability:** At LQC Level: 107.24 % At HQC level: 97.87%
Long term stability in plasma (15 days at-70°C)	**% stability:** At LQC Level: 107.84% At HQC Level: 8.78%
Dilution Integrity	**At 25% dilution:** Precision: 1.27 %Accuracy: 102.23 % **At 50 % dilution:** Precision: 2.34% Accuracy: 102.73 %

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DOI: 10.22192/ijcrcps.2018.05.04.011

How to cite this article:
Shafia Hoor F and Nagesh Babu R.. (2018). Development and Validation of High Performance Liquid Chromatographic Method for the Determination of Celiprolol in human plasma. Int. J. Curr. Res. Chem. Pharm. Sci. 5(4): 75-94.
DOI: http://dx.doi.org/10.22192/ijcrcps.2018.05.04.011