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SIMULTANEOUS ESTIMATION OF CARBODENAFIL AND DES-METHYL CARBODENAFIL FROM HUMAN PLASMA BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

A simple, sensitive and fast throughput liquid chromatography tandem mass spectrometry (LC-MS/MS) method has been developed for the simultaneous estimation of Carbodenafil and its metabolite Des-methyl Carbodenafil in human plasma, using respective deuteriated drug as internal standards. The method involved Liquid-Liquid Extraction of the analytes and internal standards from human plasma. The chromatographic separation was achieved on a ACE, CN, (150×4.6mm and 5µm particle size) analytical column using isocratic mobile phase, consisting of 5mM Ammonium Format and Acetonitrile (25:75 v/v), at a flow-rate of 1.0 mL/min with 90% flow splitting. The parent→product ion transitions 475.40→/283.20, 461.30→ /283.20, 478.40→/283.20, 469.30→/283.20 (m/z) for Carbodenafil, Des-methyl

Carbodenafil, Carbodenafil-D3 and Des-methylCarbodenafil-D8 respectively were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) positive ion mode. The method was validated over the concentration range of 2.00-1000 for Carbodenafil and Des-methyl Carbodenafil. The mean recovery values for both the drugs from spiked plasma samples were reproducible. The method was rugged and rapid with a total run time of 4.0 minutes.

KEYWORDS: *Carbodenafil*; Des-methyl Carbodenafil; LC–MS/MS; Liquid/liquid extraction.

INTRODUCTION

Carbodenafil is 5-[2-ethoxy-5-(4-ethylpiperazine-1-carbonyl) phenyl]-1-methyl-3-propyl-4H-pyrazolo [4,3-d]pyrimidin-7-oneis a type 5 phosphosiesterase (PDE5) inhibitor. It is registered for the treatment of erectile dysfunction and recently for the treatment of pulmonary hypertension. The effectiveness of Carbodenafil in the treatment of pulmonary hypertension is based on vasodilatation in well ventilated areas in the diseased lung.[1-3] After oral administration Carbodenafil is rapidly absorbed and get metabolized in the liver by CYP3A4 and is converted into the active metabolite N-Des-methyl Carbodenafil (Fig. 1). Because of its increasing popularity and potential side effects, the need for a procedure to detect both Carbodenafil and N-Des-methyl Carbodenafil in biological samples is becoming increasingly important. The simultaneous determination of Carbodenafil and the active metabolite N-Des-methyl Carbodenafil is also necessary for pharmacokinetic and related studies. Several high-performance liquid chromatographic (HPLC) methods have been reported for the determination of Carbodenafil and/or N-Des-methyl Carbodenafil in biological samples. Gas chromatography–mass spectrometry (GC/MS),[4] micellar electrokinetic chromatography,[5] liquid chromatography–mass spectrometry (LC/MS),[6,7] as well as liquid chromatography–tandem mass spectrometry (LC/MS/MS).[8–10] methods have been reported. This paper describes an analytical method for the measurement of Carbodenafil and Des-methyl carbodenafil in human serum by Liquid-Liquid extraction method without any matrix effect.

Experimental

2.1 Chemicals and materials: Working reference standards of Carbodenafil were procured from Unichem Laboratories, India as a gift sample, whereas, Des-methyl Carbodenafil, Carbodenafil-D3 and Des-methylCarbodenafil-D8 were procured from Clearsynth (p) Ltd,

India. HPLC grade methanol, acetonitrile, Analytical Reagent (AR) grade ammonium formate, Formic acid, Dichlolromethane and Diethyl ether were procured from Merck, India. Water used in the entire analysis was obtained from the in-house Milli Q water purification system. Blank human plasma was obtained from the blood bank of Supratech Micropath Laboratory, India and this drug free plasma was stored at –20°C until use.

2.2 Liquid chromatographic conditions: A Shimadzu LC system (Japan) consisting of binary gradient pumps, auto-sampler and column oven was used for setting the reverse-phase liquid chromatographic conditions. The analysis of Carbodenafil and Des-methyl Carbodenafil was performed on analytical column, ACE, CN (150x4.6mm with 5µm particle size) and maintained at 40°C in column oven. The mobile phase consists of 75% acetonitrile and 25% of 5 mM ammonium formate buffer. The flow rate of the mobile phase was kept at 1.0 mL/min with 90% flow splitting. The total chromatographic run time was 4.0min. The samples were maintained at a temperature of 5°C in the auto-sampler.

2.3 Mass Spectrometric conditions: Analyst software with version 1.4.1 was used to control all parameters of HPLC and MS. Ionization and detection of analytes and internal standards were carried out on a triple quadrupole mass spectrometer, MD Sciex API 3000 Mass Spectrometer equipped with electro spray ionization and operating in positive ion mode. Quantification was performed using selected ion monitoring (SIM) mode to monitor the parent→product ion transitions (m/z) 475.40→/283.20, 461.30→ /283.20, 478.40→/283.20, 469.30→/283.20 (m/z) for Carbodenafil, Des-methyl Carbodenafil, Carbodenafil-D3 and Des-methyl-Carbodenafil-D8 respectively. The source dependent parameters were maintained for Curtain gas (CUR) at 8.00, Temperature (TEM) at 400.00, Nebulizer gas (GS1) at 10.00, Heater gas (GS2) at----, Interface Heater (ihe) at ON and Collision gas (CAD) at 4.00. The optimum analyzer parameters are given in **Table 1**.

2.5 Analytical data processing: Peak area ratios of Carbodenafil/Carbodenafil-D3 (ISTD) and Des-methyl Carbodenafil/ Des-methylCarbodenafil-D8 (ISTD) were obtained from multiple reaction monitoring and utilized for the construction of calibration curves, using weighted ($1/x^2$) linear least squares regression of the plasma concentrations. Data collection, peak integration, and calculations were performed using Analyst software version 1.4.1. The regression equation for the calibration curve was also used to back calculate the measured concentration at each standard and control sample.

2.6 Standard stock, calibration standards and control sample preparation: The standard stock solutions of Carbodenafil and Des-methyl Carbodenafil (1 mg/mL) and Carbodenafil D3 and Des-methyl Carbodenafil (40 µg/mL) were prepared by dissolving requisite amount of drug in methanol. Diluted combined stock solution was prepared by diluting the individual stocks with methanol to obtain 100 and 50000 ng/mL of Carbodenafil and Des-methyl carbodenafil. Calibration standards and control samples were prepared by spiking in drug free blank plasma with combined stock solution. Ten calibration curve standards were made for analyte and metabolite (at 2.00, 4.00, 8.0, 12.0, 25.0, 50, 100, 200, 500 and 1000 ng/mL) while control samples were prepared at four levels (at 2.00, 6.00, 41 and 880 ng/mL). Combined internal standard stock solution of Carbodenafil-D3 and Des-methylCarbodenafil-D8 (1.0 µg/mL) was prepared by diluting Carbodenafil-D3 and Des-methylCarbodenafil-D8 stock solutions in methanol. All the aqueous solutions (standard stock, spiking solutions of calibration standards and control samples) were stored at 2–8°C and used as per the requirement of the experiments. All the plasma spiked samples were stored in deep freezer at below -20^{0}C and at below -70^{0}C and used as per the requirement of the experiments.

2.7 Sample processing: All frozen samples, calibration standards and control samples were thawed by allowing them to equilibrate to room temperature. To an aliquot of 300 µL of spiked plasma sample, 50µL of mixed ISTD dilution (1µg/mL Carbodenafil-D3 and Des-methylCarbodenafil-D8) was added to all the samples except STD Blank and vortexed for about 30 seconds. 2.5 mL of extraction solution (Diethyl ether: Dichloromethane, 70:30% v/v) was added to all the samples and extracted on rotor at 50 rpm. All the samples were centrifuged at 4000rpm for 5 minutes by using refrigerated centrifuge maintained at 10±2°C. 2.0mL of supernatant was transferred in pre-labeled tubes and evaporated the samples to dryness under nitrogen gas at about 40 ± 5°C. The dried samples were reconstituted with 100µL of Mobile Phase and vortex for about 20 seconds. All the reconstituted samples were transferred into pre-labeled auto-sampler vials, and injected in to HPLC System.

2.8 Bioanalytical method validation: Bioanalytical method validation was carried out as per the USFDA Method Validation guidelines.[11] Following parameters were evaluated during the course of Method Validation.

2.8.1 System Suitability and Auto- sampler Carryover: System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of Carbodenafil, Des-methyl Carbodenafil, Carbodenafil -D3 and Des-methyl Carbodenafil –D8

at start of each batch during the method validation. The carryover test was performed by injecting a sequence of samples consisting of aqueous standards (Drugs and ISTDs), reconstitution solution, and extracted standard (Drugs and ISTDs) equivalent to highest standard and standard blank.

2.8.2 Linearity: The linearity of the method was determined by analysis of standard plots associated with an eight point standard calibration curve. Three linearity curves containing eight non-zero concentrations were analyzed. The ratio of area response for Carbodenafil to Carbodenafil -D3 and Des-methyl Carbodenafil to Des-methyl Carbodenafil –D8 was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression which was selected and finalized during method development. Back calculations were done from these curves to determine the concentration of Carbodenafil and Des-methyl Carbodenafil in each calibration standard.

Acceptance criterion set for linearity standard were as follows

Correlation coefficient (r) for all the analytical batches should be greater than 0.99. In the lower limit of quantification (LLOQ), the analyte response should be at least five times more than the response obtained from drug free (blank) extracted plasma sample. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with a precision (%CV) not greater than 20.0 and accuracy within 80.0–120.0%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0%.

2.8.3 Selectivity: The selectivity of the method towards endogenous plasma matrix components was assessed in ten plasma lots (7 lots of normal of K3 EDTA plasma, 1 haemolysed, 1 lipidemic and 1 heparinized) of blank human plasma which were processed as per the proposed sample preparation protocol and then chromatographed to determine the extent to which endogenous plasma components may contribute towards interference at the retention time of analytes and internal standards. The cross talk of MRM for analytes and internal standards was checked using highest standard on calibration curve and working solution of internal standard.

2.8.4 Recovery: The absolute recovery of Carbodenafil, Des-methyl Carbodenafil, Carbodenafil -D3 and Des-methyl Carbodenafil –D8 was performed at low, middle and high quality control levels. It was evaluated by comparing the mean area response of five replicates of extracted samples (Blank plasma spiked with analyte followed by Liquid-Liquid

Extraction) to that of unextracted samples (Liquid-Liquid Extraction of blank plasma followed by spiking the drug to the extract) at each quality control levels. The recovery of internal standards was estimated similarly. As per the acceptance criteria, the recovery of the analytes need not be 100.0%, but should be consistent, precise and reproducible.

2.8.5 Precision and Accuracy: For determining the intra-day accuracy and precision, replicate analysis of plasma samples of Carbodenafil and Des-methyl-Carbodenafil was performed on the same day. The run consisted of a calibration curve and five replicates each of LLOQ, low, middle, high quality control samples. The inter-day accuracy and precision were assessed by analysis of three precision and accuracy batches on three consecutive validation days. The precision of the method was determined by calculating the percent coefficient of variation (%CV) for each level. The deviation at each concentration level from the nominal concentration was expected to be within ±15.0% except for LLOQ, for which the acceptance criteria is not be more than 20.0%. Similarly, the mean accuracy should not deviate by ±15.0% except for the LLOQ where it can be ±20.0% of the nominal concentration.

2.8.6 Ion Suppression: To study the ion suppression/ enhancement, the post column infusion was used during the method development. To study the effect of matrix on analytes quantification with respect to consistency in signal enhancement/ suppression, it was checked in six different lots. Six samples of LLOQ levels were prepared from six different lots of plasma and checked for the % accuracy and precision. This was assessed by comparing the back calculated value from the control samples to nominal concentration. The deviation of the standards should not be more than ±15.0% and at least 80% of the lots should be within the aforementioned criteria.

2.8.7 Stability: Stability experiments were carried out to examine the stability of analytes in stock solutions and in plasma samples under different conditions. Short term and long term stock solution stability at room temperature was assessed by comparing the area response of stability sample of analytes and internal standards with the area response of sample prepared from fresh stock solutions. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Autosampler stability, bench top stability, dry extract stability and freeze-thaw stability were performed at low and high quality control samples using three replicates at each level. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed control samples was within ±15.0.

Auto-sampler re-injection reproducibility was assessed by re-injecting one accepted precision and accuracy batch which was stored in the auto-sampler.

2.8.8 Ruggedness: To authenticate the ruggedness of the proposed method, it was done on three precision and accuracy batches. The first batch was analyzed by different analyst, second batch with different column and the third batch was analyzed on different LC-MS/MS system.

Dilution Integrity: Dilution integrity experiment was evaluated by diluting the stock solution prepared as spiked standard at concentrations of 2000 ng/mL for Carbodenafil and Des-methyl Carbodenafil. The precision and accuracy were found within ±15% from its nominal values for dilution integrity standards 1000 ng/mL after 1:2 dilution and 187 ng/mL after 1:10 dilution. Back calculated concentrations were determined by analyzing the samples against calibration curve standards.

1. RESULTS AND DISCUSSIONS

1.1 ***Mass Spectrometry:*** Carbodenafil and Des-methyl Carbodenafil and respective internal standard responded best to positive ionization and protonated molecular ions [M+H]+ were present as major peaks for the compounds. The detector was operated in multiple reaction monitoring mode (MRM). Mass transitions and compound dependent parameters are tabulated in **Table 1.**

1.2 ***Chromatography:*** **To** get best possible sensitivity and reproducibility. Various stationary phase and mobile phase compositions were tried in method development. Based on the results analytical column, ACE, CN (150x4.6mm with 5µm particle size) was chosen with mobile phase consists of 75% acetonitrile and 25% of 5 mM ammonium formate buffer.

Table 1: Analysis Condition in ESI.

Compound	Transition(m/z)	DP	EP	CE	CXP
Carbodenafil	475.40/283.20	75.0	13.4	50.2	9.6
Carbodenafil-D3	478.40/283.20	75.0	13.4	50.2	9.6
Des-methyl Carbodenafil	461.30 /283.20	65.0	10.0	45.0	9.6
Des-methyl Carbodenafil-D3	469.30/283.20	65.0	10.0	45.0	9.6

Table 2: Summary of Linearity Standards for Carbodenafil and Des-methyl Carbodenafil.

	Carbodenafil									
STD ID	**STD 10 (2.00 ng/mL**	**STD 9 (4.00 ng/mL)**	**STD 8 (8 .00 ng/mL)**	**STD 7 (12 .00 ng/mL)**	**STD 6 (25.00 ng/mL)**	**STD 5 (50.00 ng/mL)**	**STD 4 (100 .00 ng/mL)**	**STD 3 (200 .00 ng/mL)**	**STD 2 (500.00 ng/mL)**	**STD1 (1000 ng/mL)**
Mean	1.98	3.99	8.18	12.3	25.3	50.4	101	199	490	964
SD	0.106	0.151	0.273	0.303	0.459	1.10	2.30	6.09	12.5	22.8
%CV	5.35	3.78	3.34	2.46	1.81	2.18	2.28	3.06	2.55	2.37
% Bias	-1.00	-0.25	2.25	2.50	1.20	0.80	1.00	-0.50	-2.00	-3.60
	Des-methyl Carbodenafil									
STD ID	STD 10 (2.00 ng/mL	STD 9 (4.00 ng/mL)	STD 8 (8 .00 ng/mL)	STD 7 (12 .00 ng/mL)	STD 6 (25.00 ng/mL)	STD 5 (50.00 ng/mL)	STD 4 (100 .00 ng/mL)	STD 3 (200 .00 ng/mL)	STD 2 (500.00 ng/mL)	STD1 (1000 ng/mL)
Mean	1.98	4.03	8.06	12.3	25.1	50.0	99.8	198	496	987
SD	0.0617	0.0891	0.206	0.404	0.603	0.959	1.94	6.88	10.4	26.2
%CV	3.12	2.21	2.56	3.28	2.40	1.92	1.94	3.47	2.10	2.65
% Bias	-1.00	0.75	0.75	2.50	0.40	0.00	-0.20	-1.00	-0.80	-1.30

Table 3: Intra-day and Inter-day quality control samples for Carbodenafil and Des-methyl Carbodenafil.

QC	**Carbodenafil**				**Des-methyl Carbodenafil**			
Intra-batch	**LLOQ QC (2 ng/mL**	**LQC (6 ng/mL)**	**MQC (41ng/mL)**	**HQC (880 ng/mL)**	**LLOQ QC (2 ng/mL)**	**LQC (6 ng/mL)**	**MQC (41 ng/mL)**	**HQC (880 ng/mL)**
Mean	1.88	6.12	42.0	852	1.97	6.30	41.1	851
SD	0.0691	0.137	1.31	6.04	0.0555	0.140	1.97	14.1
%CV	3.68	2.24	3.12	0.71	2.82	2.22	4.79	1.66
% Bias	-6.00	2.00	2.44	-3.18	-1.50	5.00	0.24	-3.30
Mean	2.00	5.90	39.5	807	2.10	5.90	40.0	827
SD	0.123	0.285	0.828	18.0	0.0829	0.176	0.773	23.5
%CV	6.15	4.83	2.10	2.23	3.95	2.98	1.93	2.84
% Bias	0.00	-1.67	-3.66	-8.30	5.00	-1.67	-2.44	-6.02
Mean	1.88	6.17	40.7	868	1.96	6.04	40.6	876
SD	0.0844	0.116	0.559	6.06	0.0277	0.177	0.259	18.3
%CV	4.49	1.88	1.37	0.70	1.41	2.93	0.64	2.09
% Bias	-6.00	2.83	-0.73	-1.36	-2.00	0.67	-0.98	-0.45
Inter-batch								
Mean	1.92	6.06	40.7	842	2.01	6.08	40.6	851
SD	0.106	0.218	1.36	28.8	0.0852	0.230	1.23	27.2
%CV	5.52	3.60	3.34	3.42	4.24	3.78	3.03	3.20
% Bias	-4.00	1.00	-0.73	-4.32	0.50	1.33	-0.98	-3.30

Table 4: Stability of Carbodenafil and Des-methyl Carbodenafil in Human plasma at two QC levels (n=5).

Stability Condition	Compound	Nominal Concentration (ng/mL)	Calculated concentration	
			Mean ± SD	% Bias
Bench Top stability	Carbodenafil	6	6.28±0.287	4.67
		880	858±15.00	-2.50
	Des-methyl Carbodenafil	6	6.05±0.155	0.83
		880	855±15.00	-2.84
Wet extract Stability	Carbodenafil	6	5.61±0.307	-6.50
		880	789±34.30	-10.34
	Des-methyl Carbodenafil	6	5.86±0.156	-2.33
		880	819±26.3	-6.93
Freeze thaw stability after 5 cycles at -20°C	Carbodenafil	6	5.92±0.344	-1.33
		880	839±25.20	-4.66
	Des-methyl Carbodenafil	6	6.02±0.0416	0.33
		880	848±5.29	-3.64
Freeze thaw stability after 5 cycles at - 78°C	Carbodenafil	6	6.15±0.100	2.50
		880	837±8.50	-4.89
	Des-methyl Carbodenafil	6	6.01±0.0854	0.17
		880	844±3.06	-4.09
Auto-sampler Stability	Carbodenafil	6	6.17±0.162	2.83
		880	836±8.29	-5.00
	Des-methyl Carbodenafil	6	6.22±0.130	3.67
		880	877±18.4	-0.34
Long Term Stability in Plasma at -20°C	Carbodenafil	6	6.25±0.640	4.17
	Des-methyl Carbodenafil	880	952±58.6	3.07
		6	5.92±0.281	-1.33
		880	907±29.0	3.07
Long Term Stability in Plasma at -78°C	Carbodenafil	6	6.73±0.163	12.17
		880	940±31.6	6.82
	Des-methyl Carbodenafil	6	5.97±0.124	-0.50
		880	887±23.9	0.80

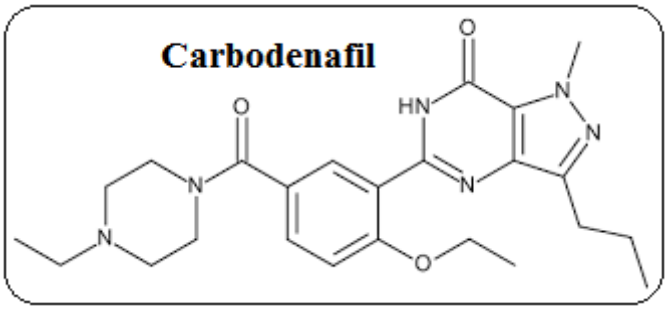

Figure 1: Structure of Carbodenafil.

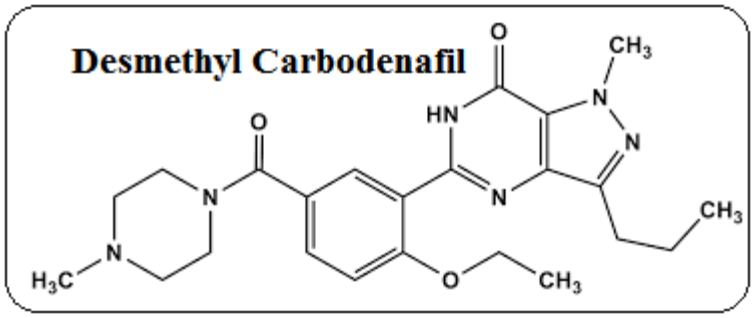

Figure 2: Structure of Des-methyl Carbodenafil.

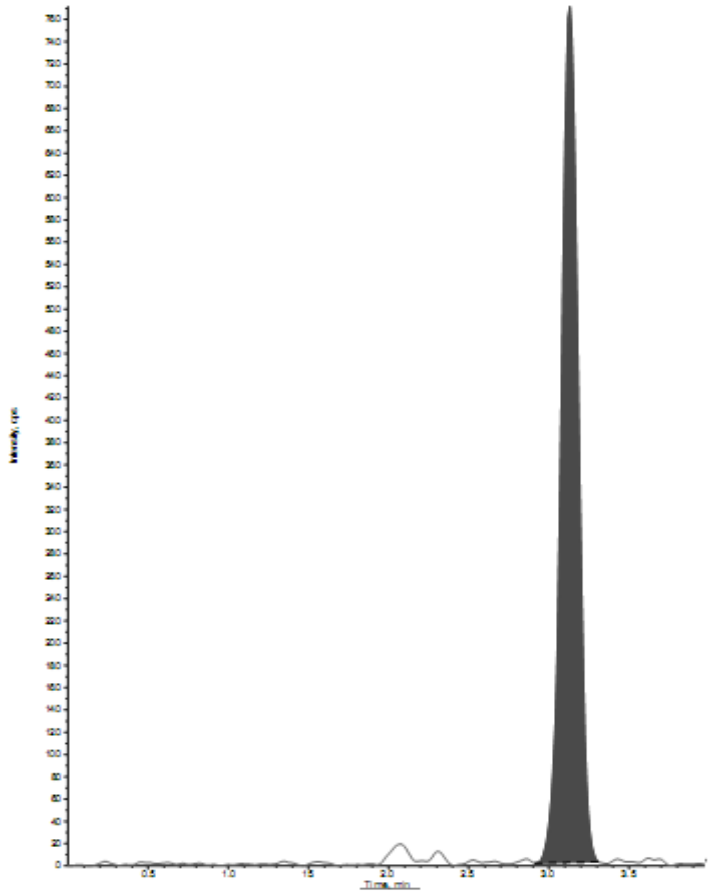

Figure 3: Representative Chromatogram of LLOQ (2 ng/mL) Sample of Carbodenafil.

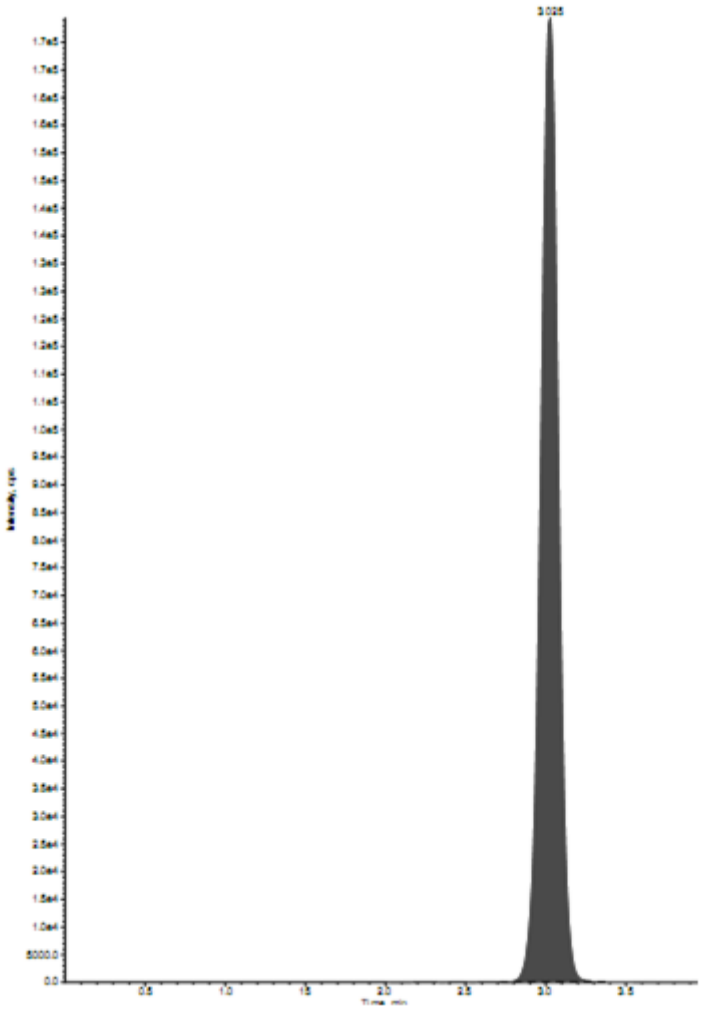

Figure 4: Representative Chromatogram of LLOQ (2 ng/mL) Sample of Des-methyl Carbodenafil.

3.3 Method validation

3.3.1 **Linearity and Lower Limit of Quantification (LLOQ):** All the three calibration curves analyzed during the course of validation were linear for the standards ranging from 2.00 to 1000 ng/mL. A straight-line fit was made through the data points by least square regression analysis and a constant proportionality was observed. In order to establish the best weighting factor back-calculated calibration concentration was determined. The model with the lowest total bias and most consistent bias across the range was considered as the best fit. Weighting factor of 1/x2 was giving best possible results. Using weighted least squares with weights that are inversely proportional to the variance at each level of the explanatory variables yields the most precise parameter estimates possible. The mean values for slope, intercept and correlation coefficient (r) observed during the course of validation were 0.0060, 0.0018 and 0.9982 for Carbodenafil and 0.0062, 0.0023 and 0.9988 for Des-methyl

Carbodenafil respectively. The %bias and precision (%CV) observed for the calibration curve standards was -3.60 to 2.25 and ≤ 5.35 for Carbodenafil and -1.30 to 2.50 and ≤ 3.47 for Des-methyl Carbodenafil respectively. The lower limit of quantification (LLOQ) achieved was 2.00 ng/mL for Carbodenafil and for Des-methyl Carbodenafil. The mean bias (%) for back calculated concentration of LLOQ was -1.00 with precision (%CV) of 5.35 for Carbodenafil and the mean bias (%) for back calculated concentration was -0.10 with precision (%CV) of 3.12 for Des-methyl Carbodenafil. **Table 2** summarizes the mean back calculated concentration with % bias and precision data for all the fourteen linearity curves.

3.3.2 Selectivity, Recovery, Precision and Accuracy (Bias)

The selectivity of the method towards endogenous plasma matrix was ascertained in six batches of human plasma by analyzing blanks and spiked plasma samples at LLOQ concentration. No endogenous peaks were observed at the retention time of the analytes for any of the batches. **Fig. 3&4.**

Five replicates each at low, middle and high levels were prepared for recovery determination. The % mean recovery was 83.0% and 76.4% for Carbodenafil and Des-methyl Carbodenafil respectively. The recovery of internal standards, Sildenail-D3 and Des-methyl Carbodenafil-D8 was 76.4% and 76.4% respectively. The intra-batch and inter-batch accuracy and precision was determined in three batches at LLOQ, low, middle and high levels with six replicates for each batch. For Carbodenafil, the precision (%CV) for intra batch and inter batch is < 6.15 and < 5.52 respectively for all control samples. For Des-methyl Carbodenafil, the precision (%CV) for intra batch and inter batch is < 4.79 and < 4.24 respectively for all control samples. For Carbodenafil, the % bias for intra batch ranged from -8.3.00 to 2.83 and for inter-batch bias was from –4.30 to 1.00. For Des-methyl Carbodenafil, the % bias for intra batch ranged from -6.02 to 5.00 and for inter-batch bias was from -3.30 to 1.33. The detailed results are presented in **Table 3**.

3.3.3 Matrix effect and Stability

Matrix effect is due to co-elution of some components present in biological samples. These components may not give a signal in MRM of target analytes but can certainly decrease or increase the analytes response dramatically to affect the sensitivity, accuracy and precision of the method. Thus assessment of matrix effect constitutes an important and integral part of validation for quantitative LC-MS/MS method for supporting pharmacokinetics studies. No significant signal suppression/enhancement was observed due to endogenous plasma matrix

at the retention times of Carbodenafil, Des-methyl Carbodenafil, Carbodenafil –D3 and Des-methyl Carbodenafil –D8 using post column infusion. The % mean accuracy of back calculated concentration for LLOQ samples from six different matrix lots was 93% with precision of 6.83% for Carbodenafil and 96.92% with precision of 2.58% for Des-methyl Carbodenafil.

Stock solutions of Carbodenafil, Des-methyl Carbodenafil, Carbodenafil –D3 and Des-methyl Carbodenafil were stable at room temperature for minimum period of 8.0 hours and when stored between 2-8°C they were stable for 81 days. Carbodenafil, Des-methyl Carbodenafil, Carbodenafil –D3 and Des-methyl Carbodenafil in control human plasma (bench top) at room temperature was stable for at least 8.0 hours at 25°C and for minimum of five freeze and thaw cycles at temperatures -20^{0}C and -78^{0}C. Autosampler stability of the spiked control samples maintained at 5^{0}C was determined up to 27.0 hours. Long term stability of the spiked control samples stored at -78^{0}C was found stable for 73 days. Different stability experiments in plasma and the values for the precision and percent change are shown in **Table 4**.

3.3.4 Ruggedness and Dilution Integrity

Ruggedness was performed by using three precision and accuracy batches. The first batch was analyzed by different analyst, the second batch was analyzed on different column and the third batch was analyzed on different equipment.

For all the experiments for Carbodenafil, the precision was ≤ 8.39 and and for Des-methyl Carbodenafil, the precision was < 5.73% which is within the acceptance limit of 15%.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analytes concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis.

For Carbodenafil, the precision for dilution integrity of 1/2 and 1/10th dilution were 1.61 and 1.05%, while the bias was -6.00 and -1.50% respectively and for Des-methyl Carbodenafil, the precision for dilution integrity of 1/2 and 1/10th dilution were 1.84 and 1.34%, while the bias was -3.00 and 0.50% respectively, which is within the acceptance limit of 15% for precision (CV) and ± 15% of bias.

CONCLUSIONS

The bio-analytical methodology for determination of Carbodenafil and Des-methyl Carbodenafil described in this manuscript is highly specific, rugged and rapid for therapeutic drug monitoring both for analysis of routine samples of single dose or multiple dose pharmacokinetics and also for clinical trial samples with desired sensitivity, precision, accuracy and high throughput. The method involved a simple and specific sample preparation by solid phase extraction followed by isocratic chromatographic separation in 2.0 min. The overall analysis time is promising compared to other reported procedures for Carbodenafil and Des-methyl Carbodenafil. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with any marketing formulation of Carbodenafil and Des-methyl Carbodenafil in human volunteers.

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