Development and application of Liquid Chromatographic Method for Determination of Clebopride in Bulk and in Tablet Dosage forms

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Abstract- *Clebopride is an antifungal agent of the novel echinocandin class. Clebopride, the first inhibitor of fungal b-1,3 glucan synthesis to receive approval by the United States Food and Drug Administration, is effective for the treatment of mucosal and invasive candidiasis and invasive aspergillosis. It is also active in vitro and in animal models against a number of other filamentous and dimorphic endemic fungi and in animal models of Pneumocystis carinii infection. Clebopride is a water-soluble amphipathic lipopeptide is a semisynthetic derivative of pneumocandin B0, a fermentation product of Glarea lozoyensis. Developing a accurate and precise alytical method for the estimation of cagpsofungin in a sterile, lyophilized product for intravenous (IV) infusion a is very challenging, due to the formation of drug-drug and drug-excipient interactions. The present study demonstrates the applicability of chromatographic method to develop a new, sensitive, single HPLC method for the quantitative determination of antifungal agents in freeze dried powder for injection pharmaceutical dosage form. Chromatographic separation active pharmaceutical ingredient was achieved by using a isocratic elution at a flow rate of 1.0 mL/min on X-Terra RP-18 column (250mm×4.6 mm, 5µm particle size, 100Å pore size) at ambient temperature. The contents of the mobile phase were 3.48 gms of Di Potassium hydrogen ortho-phosphate (0.03M) in 1000 ml of water and by adjusting the pH to 3.2 with dilute ortho-phosphoric acid (mobile phase solvent-A) and methanol (mobile phase solvent-B) in a isocratic mode in the ratio of 30: 70 (v/v) of separation was used to resolute the Clebopride. UV detection at 225 nm was employed to monitor the analytes. A linear response was observed for Clebopride over the concentration range 0.5–6 µg/ mL. Limit of detection (LOD) and Limit of quantification (LOQ) for Clebopride were found to be 0.025µg/mL, and 0.075µg/mL respectively.*

Keywords- Clebopride, Isocratic-HPLC, Casporan®, Lyophilized powder for injection.

I. INTRODUCTION

Clebopride (Fig. 1), 4-amino-N-(1-benzylpiperidin-4-yl)-5-chloro-2-methoxybenzamide, is a dopamine antagonist drug with antiemetic and prokinetic properties used to treat functional gastrointestinal disorders. Detailed investigation at several centers has demonstrated its encouraging antiemetic, gastrokinetic and anxiolytic properties.1–3Literature survey denotes that the drug can be estimated by thin-layer chromatography and high-performance liquid chromatography,4,5 UV spectrophotometry6 gas chromatography-mass spectrometry and radioimmunoassay in both animals7 and man.8,9 In the present work, an attempt has been made to develop and validate a simple RP-HPLC method for the analysis of clebopride from human plasma.

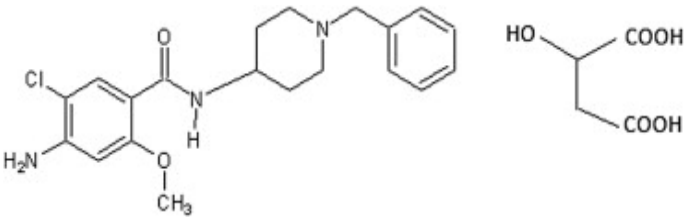

Figure 1. Chemical structures of Clebopride

II. EXPERIMENTAL

Chemicals and reagents:

Clebopride was obtained as kind gift sample from shantha Biotech Ltd, Hyderabad. Potassium dihydrogen ortho-phosphate, methanol and ortho-phosphoric acid were obtained from Merck, Mumbai, India. All the solutions were prepared in Milli Q water (Millipore, USA). Test samples composed of Clebopro® 0.2 mg film coated tablets, Bayer, India contains 0.2 mg of Clebopride, is obtained from local market.

HPLC Instrumentation and Chromatographic conditions:

Quantitative HPLC was performed on the Waters Alliance 2695 Separations Module is a high performance liquid chromatographic system with a quaternary, low-pressure mixing pump and inline vacuum degassing. Waters

Alliance 2695 separation module (Waters Corporation, Milford, USA) equipped with 2489 UV/visible detector or 2998 PDA detector with Empower 2 software was used for the analysis. Flow rates from 50 uL/ min to 5 mL/min can be generated for use with 2.1 mm ID columns and larger. The auto-sampler has a maximum capacity of 120 vials (12x32, 2-mL) with programmable temperature control from 4 to 40°C. A heated column compartment provides temperatures from 5 degrees above ambient to 65°C. The detector is a photodiode array (model 2996) with a wavelength range of 190-800 nm and sensitivity settings from 0.0001-2.0000 absorbance units The HPLC system was equipped with a column compartment with temperature control and an on-line degasser. X-Terra RP-C18 Column (250x4.6 mm i.d; particle size 5 µm) was used for separation of Clebopride. The contents of the mobile phase were 3.48 gms of Di Potassium hydrogen ortho-phosphate (0.03M) in 1000 ml of water and by adjusting the pH to 3.2 with dilute ortho-phosphoric acid (mobile phase solvent-A) and methanol (mobile phase solvent-B) in a isocratic mode in the ratio of 35: 65 (v/v) of separation was used to resolute the Clebopride. They were filtered before use through a 0.45 µm membrane filter and degassed by sonication. The flow was adjusted at 1.0 ml/min flow rate and 20 µL injection load volumes were maintained. The eluted compounds were monitored at 225 nm. The column oven temperature was maintained at 25◦C. Data acquisition, analysis, and reporting were performed by Empower2 (Waters) chromatography software.

III. PREPARATION OF SOLUTIONS

Standard and stock solutions: Standard solution of the active pharmaceutical ingredient was prepared in the following manner: Transfer 50 mg of Clebopride working standard into a 100 ml volumetric flask, dissolve and dilute with Methanol and water in the ratio of 50:50 v/v as diluent. 5 ml of the resulting solution is further diluted up to 50 ml in volumetric flask with diluents. The resulting solution contains 50 µg/mL of Clebopride as working standard solutions. The prepared stock solutions were stored at 4 0C and protected from light.

Preparation of the Sample solution: Clebopro® (Clebopride sodium tablets) is supplied as tablets containing 0.5 mg of Clebopride sodium, for oral administration. Active Ingredient: Clebopride sodium. 20 Clebopro® 0.5 mg film coated tablets were collected, their average weight was recorded. Then they are crushed to fine homogenous powder, uniformly blended and a quantity equivalent to 5 mg was weighed and transferred in to a 10-mL volumetric flask, extracted in diluent by sonication, and filtered through Whatman no. 41 filter paper. The filtrate (5 mL) was quantitatively transferred to a 50-mL volumetric flask, and solution was diluted to volume with the diluents. The resulting solution contains 50 µg/mL of Clebopride as working sample solutions. The prepared stock solutions were stored at 4 0C and protected from light.

IV. SOLUTIONS FOR VALIDATION STUDY

Calibration and Quality control samples: Calibration standards (5–60 µg/ mL of Clebopride were prepared from working standard solutions by appropriate dilution with Methanol and water in the ratio of 50:50 v/v as diluents. Quality control (QC) samples for accuracy studies were prepared at three concentrations of the linearity range (40 µg/ mL, 50 µg/ mL and 60 µg/ mL) for Clebopride were prepared from the standard solutions.

Method Validation: The developed chromatographic method was validated for selectivity, linearity, precision, accuracy, sensitivity, robustness and system suitability.

Specificity: The terms selectivity and specificity are often used interchangeably. The specificity of the developed LC method for quantification of active pharmaceutical ingredient was determined the presence of excipients present in pharmaceutical products. In specificity study, interference between drugs and excipients usually employed in film coated tablets were evaluated from the comparison of spectral purity obtained from the analysis for the standard solutions and sample solutions.

System suitability: The system suitability was assessed by six replicate analyses of the drugs at concentrations of 50 µg/ mL for Clebopride. The acceptance criterion was ±2% for the RSD for the peak area and retention times for all four analytes. The system suitability parameters with respect to theoretical plates, tailing factor, repeatability and resolution between peak and peaks of the other three analytes were defined.

Linearity: Linearity of the method was evaluated at seven equi-spaced concentration levels by diluting the standard solutions to give solutions over the ranges 10–120% target concentration for main analyte of interest. The calibration curves were constructed at seven concentrations between 5–60 µg/ mL for Clebopride. These were injected in triplicate and the peak areas were inputted into a Microsoft Excel® spreadsheet program to plot calibration curves. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method. The peak areas of the analyte to concentration of analyte were used for plotting the linearity graph. The linearity data is reported in Table-3.

Table 3. Linearity Data for Clebopride

Precision: Precision was evaluated in terms of intra-day repeatability and inter-day reproducibility. The intra-day repeatability was investigated using six separate sample solutions prepared, as reported above, from the freshly reconstructed tablet formulations at 100% of the target level. Each solution was injected in triplicate and the peak areas obtained were used to calculate means and RSD% values. The inter-day reproducibility was, by preparing and analyzing in triplicate sample solutions from the reconstructed formulations at the same concentration level of intra-day repeatability; the means and RSD% values were calculated from peak areas. (Table-4)

Table 4. Intra-day and inter-day precision data for Clebopride

Accuracy: The accuracy of the method was determined by measuring the recovery of the drug by the method of standard additions. Quality control (QC) samples for accuracy studies were prepared at three concentrations of the linearity range (40 µg/ mL (80% dilution), 50 µg/ mL (100% dilution) and 60 µg/ mL (120% dilution) for Clebopride were prepared from the standard solutions. Known amounts of 10 % dilution of drug (5 µg/mL of Clebopride) was added to corresponding to 80%, 100%, and 120% of the target test concentrations were added to a placebo mixture to determine whether the excipients present in the formulation led to positive or negative interferences. Each set of additions was repeated three times at each level. Extraction sample preparation procedure is followed and assayed against qualified reference standard. The accuracy was expressed as the percentage of the analytes recovered by the assay. (Table-5)

Table 5. Accuracy: recovery data for Clebopride

Sensitivity: Limits of detection (LOD) and quantification (LOQ) were estimated from the signal- to-noise ratio. The detection limit was determined as the lowest concentration level resulting in a peak area of three times the baseline noise. The limit of detection was determined, by injecting progressively low concentrations of analyte of interest. The quantification limit was determined as the lowest concentration level that provided a peak area with signal-to-noise 10.

Robustness: To determine the robustness of the developed method, experimental conditions were deliberately changed and the relative standard deviation for replicate injections of Clebopride and the USP resolution factor between and the other two peaks were evaluated. The mobile phase flow rate was 1.0 mL/min. This was changed by ±0.2 units to 0.8 and 1.2 mL/min. The effect of stationary phase was studied by the use of LC columns from different batches at 25°C. The effect of buffer pH was studied at pH 3.0 and 3.4 (± 0.2 units). The chromatographic variations were evaluated for resolution between and the other three analytes in a system suitability solution with respect to retention time RT and % assay of drugs.

Table-6: Robustness data for Clebopride

Solution stability: To assess the solution stability, standard and test solutions were kept at 25 ◦C (laboratory temperature) for 24 h. These solutions were compared with freshly prepared standard and test solutions.

V. RESULTS AND DISCUSSION:

HPLC method development: The API solution of analyte of interest i,e., Clebopride was prepared in diluent at a concentration of 50µg/mL and scanned in UV-Visible spectrometer; and the Clebopride was found to have UV maxima at around 225 nm. Hence detection at 225 nm was selected for method development purpose. Some important parameters, pH of the mobile phase, concentration of the acid or buffer solution, percentage and type of the organic modifier, etc. were tested for a good chromatographic separation. The main analytical challenge during development of a new method was obtaining adequate retention of the polar compound Clebopride. Trials showed that acidic mobile phase with reverse phase column gives symmetric and sharp peaks. For this reason, potassium dihydrogen phosphate buffer with pH-3.2 was adjusted with o-phosphoric acid was preferred as acidic buffer solution. Methanol and buffer in the ratio of 65:35 (v/v) were chosen as the organic modifier because it dissolves drugs very well. Mobile phase composition in isocratic mode at a flow rate of 1.0 mL per minute was observed for a good resolution. Then method was optimized to separate the active ingredient by changing to isocratic mode. The satisfactory chromatographic separation, with good peak shapes were achieved on X-Terra RP-18-C18 (250 × 4.6) mm with 5 µm particles, using the column temperature as maintained at 35°C and the detection was monitored at a wavelength of 225 nm. The injection volume was 20 µL. Methanol and water in the ratio of 50:50 v/v) were used as diluent. In the optimized isocratic conditions, Clebopride was well separated with a resolution (Rs) of greater than 2 and the typical retention time of about 2.835 minutes, the typical chromatogram of System suitability shown in Figure 2.

Method validation:

The developed method was validated, as described below, for the following parameters: system suitability, selectivity, linearity, precision, accuracy and LOD/LOQ.

Selectivity: Selectivity of the current method was demonstrated by good separation of the active ingredients.

Furthermore, matrix components, e.g. excipients, do not interfere with the four analytes as they have no absorbance. The representative chromatogram (Fig. 5A) of the tablet dosage form solution containing excipients showed no peak interfering with analytes; moreover the adjacent chromatographic peak was separated with resolution factors >3. Overall, these data demonstrated that the excipients did not interfere with the active ingredients peaks, indicating selectivity of the method

System suitability: The RSD values of peak area and retention time for the analytes are within 2% indicating the suitability of the system.

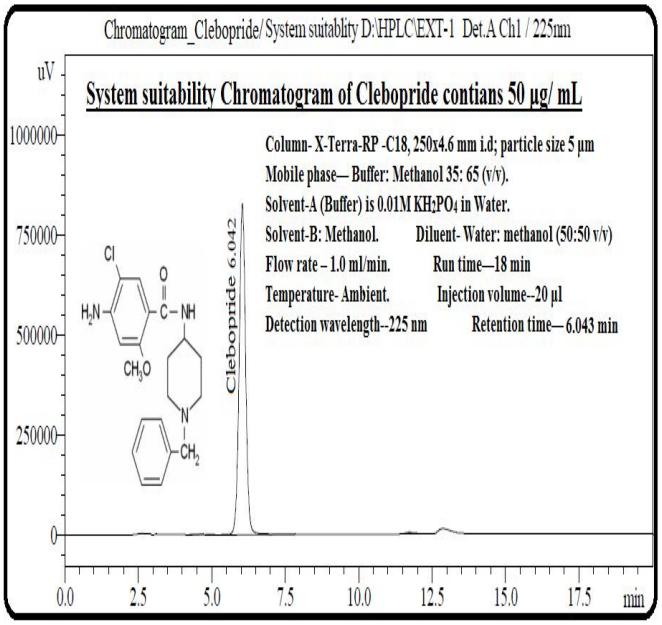

Figure 2. System suitability chromatogram of working standard solution contains 20 µg/mL of Clebopride.

Table 2. Results of System suitability study

Parameter	Clebopride
Retention time	6.042
Theoretical plates	7405.469
Tailing Factor	1.024
HETP	29621.88
USP plates/meter	$3.375x10^{-5}$
Resolution	3.060
Peak area	12706026
% of Peak area	99.496

Linearity and range: Seven concentration levels within 10–120% of the target concentration range for analytes were considered to study the linearity. The calibration curves were prepared by plotting the peak area of the drug to the respective concentrations, which were linear in the range of 5–60 µg/ mL for Clebopride. Peak areas of the active ingredients and concentrations were subjected to least square linear regression analysis to calculate the calibration equations and correlation coefficients. The mean regression equations were found as Y= 218635.584 x+ 351360.4873 for Clebopride. The square of the correlation coefficient (r2 > 0.999) demonstrated a significant correlation between the concentration of analytes and detector response. The results show that there is an excellent correlation between the peak area ratios and the concentrations of drugs in the range tested.

Table 3. Linearity data for the Clebopro®- 0.5 mg- Film coated tablets

Concentration	Peak Area	Parameter	Clebopride
5 µg/ mL	1218810	Concentration Range	5-60 µg/ mL
10 µg/ mL	2791563	Regression equation	Y= 218635.584 x+ 351360.4873
20 µg/ mL	4753532	Correlation Coefficient	0.999
30 µg/ mL	6844398	0.95 Confidence interval	Lower-Limit-0.993/ Upper Limit-1
40 µg/ mL	9139549	0.95 Confidence interval	Lower-Limit-0.987/ Upper Limit-1
50 µg/ mL	11250133	Limit of Detection(LOD)	0.025 µg/ mL
60 µg/ mL	13468189	Limit of Quantification(LOQ)	0.075 µg/ mL

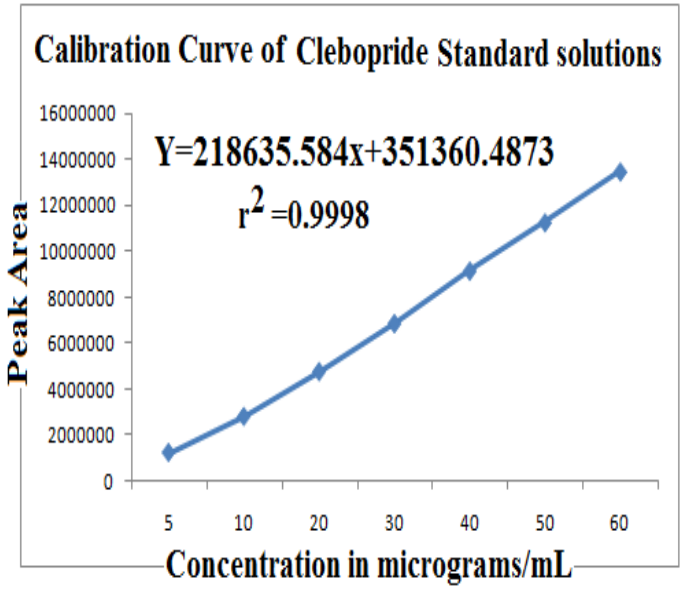

Figure 3. Calibration Curve of Clebopro® 0.5 mg- Film coated tablets.

Precision: Precision of this method was determined by injecting the standard solution of the three analytes six times.

The R.S.D. of peak area of six replicates was found to be less than 2. The results obtained are shown in Table 4. In all instances the %RSD values were less than 2%.

Table 4. Intra-day and inter-day precision data for Clebopride

Precision data of Clebopride	**Inter-day precision**		**Intra-day precision**	
Analyte-conc. (20 µg/ml)	Retention time in min.	Peak Area	Retention time in min.	Peak Area
Clebopride injection-1	5.655	11081035	5.533	10939024
Clebopride injection-2	5.522	11076339	5.643	11004495
Clebopride injection-3	5.478	11109892	5.501	11017949
Clebopride injection-4	5.647	11103533	5.494	11047133
Clebopride injection-5	5.838	11024969	5.602	11068241
Clebopride injection-6	5.521	11068769	5.508	10975878
Mean	5.610	11077423	5.547	11008787
% RSD.	2.371	0.273	1.108	0.427
Std. Deviation	0.133	30254	0.061	47026

Accuracy: Percentage recovery of the active ingredient using this method was determined using Clebopro® 0.5 mg is (Clebopride sodium tablets) is supplied as tablets containing 0.5 mg of Clebopride sodium, for oral administration. The results of accuracy studies from standard solution and excipient matrix were shown in Table 5; recovery values demonstrated that the method was accurate within the desired range.

Table 5. Accuracy study and recovery data for Clebopride

S. No	Recovery at 80% dilution Level Peak areas		Recovery at 100% dilution Level Peak areas		Recovery at 120% dilution Level Peak areas	
	Standard	Spiked	Standard	Spiked	Standard	Spiked
1	89590169	10210497	11080307	12250313	13696646	14355100
2	90400858	10189861	11086282	12277354	13444471	14287788
3	91000682	10178981	11025259	12525949	13544459	14217074
Avg	90335570	10193113	11063949	12351205	13561859	14286654
Std. Dev	71037	16008	33640	15193 5	126985	69020
%RSD	0.786	0.157	0.304	1.230	0.936	0.483
% Recovery	104.65		106.4		98.97	

Clebopro® sterile, lyophilized product for intravenous (IV) infusion working sample solution was spiked
-at 80% level (40 µg/ml was spiked with 10% of mixed standard solution of API's(5 µg/ml)
-at 100% level (50 µg/ml was spiked with 10% of mixed standard solution of API's(5 µg/ml)
-at 120% level (60 µg/ml was spiked with 10% of mixed standard solution of API's(5 µg/ml)

Sensitivity: Limit of detection (LOD) for Clebopride was 0.025 µg/mL and limit of quantification (LOQ) for Clebopride was 0.075µg/mL. The results of LOD and LOQ were indicating a high sensitivity of the method.

Robustness: The HPLC parameters were deliberately varied from normal procedural conditions including the mobile phase flow rate was 1.0 mL/min. This was changed by ±0.2 units to 0.8 and 1.2 mL/min. The effect of stationary phase was studied by the use of LC columns from different batches at

35°C. Under these variations, all analytes were adequately resolved and elution orders remained unchanged. The testing solution maintained a signal-to-noise ratio over 10 in all varied conditions. The peak resolution was all larger than 1.5 under each variation.

Table 6. Robustness study of Clebopro® 0.2 mg Film coated tablet solution at 100 % level (20 µg/mL)

Parameter	**Clebopride** in Flow increase study		**Clebopride** in Flow decrease study		**Clebopride** in Variable column Study	
	Run time	Peak Area	Run time	Peak Area	Run time	Peak Area
Injection-1	5.512	10360298	6.709	12437512	5.503	11069769
Injection-2	5.475	10393734	6.692	12438174	5.490	11118461
Injection-3	5.492	10299095	6.682	12397454	5.491	11125822
Mean	5.493	10351042	6.695	12424380	5.495	11104684
% RSD	0.335	0.464	0.203	0.188	0.131	0.274
Std. Dev	0.018	47993	0.014	23321	0.007	30461

VI. Analysis of the film coated tablet

Clebopro® (Clebopride sodium tablets) is supplied as tablets containing 0.5 mg of Clebopride sodium, for oral administration. Active Ingredient: Clebopride sodium. Inactive Ingredients: mannitol, magnesium stearate, sodium hydroxide, crospovidone, povidone, iron oxide yellow, methylhydroxypropylcellulose, polyethylene glycol, and titanium dioxide. 20 Clebopro® 0.5 mg film coated tablets were collected, their average weight was recorded. Then they are crushed to fine homogenous powder, uniformly blended to obtain fine tablet powder. An amount of the homogenous powder equivalent to 5 mg was transferred into a 100ml volumetric flask, added 40 ml of diluents (Methanol and water in the ratio of 50:50 v/v), sonicated for 30 min, diluted to 100 ml with diluents. 50ml sample taken from this solution was centrifuged at 3000 rpm for 15 min. Test solutions were then made up to volume with the diluent. The filtrate (5 mL) was quantitatively transferred to a 50-mL volumetric flask, and solution was diluted to volume with the diluents. The resulting solution contains 50 µg/mL of Clebopride as working sample solutions. The prepared stock solutions were stored at 4 0C and protected from light. The amount of Clebopride in standard mixtures or dosage forms were individually calculated using the related linear regression equations.

On the basis of above results, the proposed method was applied to the determination of antifungal agent Clebopride present in tablet dosage forms. Figure-3 shows representative chromatograms obtained from the analysis of Clebopro® 0.5 mg (Clebopride sodium tablets). The differences between the amount claimed and those assayed were very low and the R.S.D. values were within the acceptable range mentioned by pharmacopoeias. The mean percentage recoveries obtained after six repeated experiments were found between 98 and 108.2 (Table 6), indicating that the results are accurate and precise and there is no interference from the common excipients used in the pharmaceutical dosage forms.

Table 7. Assay results of Clebopro®- 0.2 mg- Film coated tablets

Formulation	Label Claim (mg/tablet)	Amount found in (mg/ tablet)
Tablet	0.5	0.495

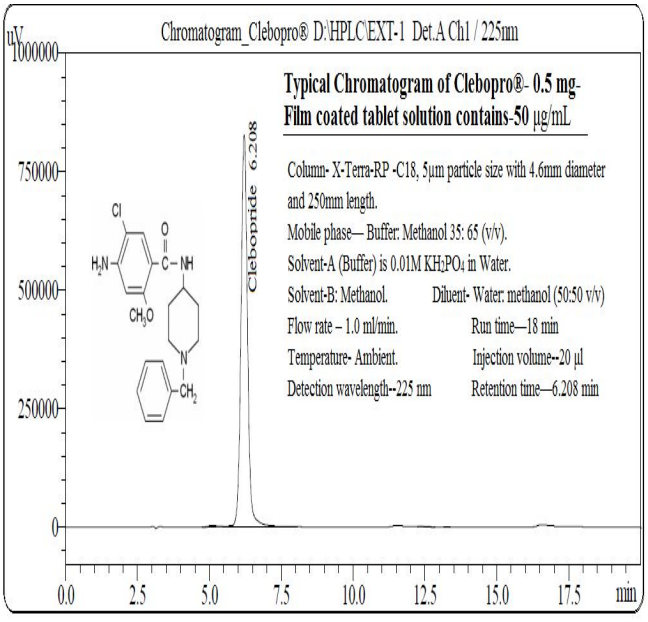

Figure 5. Typical Chromatogram of Clebopro®- 0.5 mg- Film coated tablets

VII. CONCLUSION

In this study, a validated simple and reliable RP-HPLC-PDA procedure was described for the assay of a Clebopro®-0.5 mg film coated tablets that contains a Clebopride, which is indicated is indicated as empirical therapy for presumed fungal infections in febrile, neutropenic adult and pediatric patients. To our present knowledge, no attempts have yet been made to estimate these tablets by analytical procedure. The active pharmaceutical ingredient was successfully resolved and quantified using X-Terra RP-18 Octadecyl column (250×4.6mm, 5µm) in a relatively short run time of 18 minutes in isocratic mode s chromatographic method. The proposed method provides a good resolution between active ingredients. The developed method reported herein was validated by parameters as described in ICH-Q2B guideline. System suitability, specificity, linearity, LOD, LOQ values, within- and between-day precision and accuracy of the proposed technique were obtained during the validation studies. The proposed method has the advantages of simplicity, repeatability, sensitivity and requires less expensive reagents.

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