Scientific Journal of Impact Factor (SJIF): 5.71

e-ISSN (O): 2348-4470
p-ISSN (P): 2348-6406

International Journal of Advance Engineering and Research Development

Volume 5, Issue 03, March -2018

Development and application of Liquid Chromatographic method for determination of Carvoverine in bulk and in Intramuscular injection dosage forms

Dr.L.Satyanarayana*, Sumalatha. Reddi

Department Of Pharmaceutical Analysis, Omega College Of Pharmacy, Ghatkesar, Hyderabad, T.S.500034

Abstract: *Carvoverine is an antifungal agent of the novel echinocandin class. Carvoverine, 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. Carvoverine 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 Gradient elution at a flow rate of 1.0 mL/min on Zorbax Eclipse XDB-C18 (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 Acetonitrile (mobile phase solvent-B) in a Gradient mode in the ratio of 30: 70 (v/v) of separation was used to resolute the Carvoverine. UV detection at 260 nm was employed to monitor the analytes. A linear response was observed for Carvoverine over the concentration range 4-48 µg/ mL. Limit of detection (LOD) and Limit of quantification (LOQ) for Carvoverine were found to be 0.04µg/mL, and 0.12µg/mL respectively.*

Key words: *Carvoverine, Gradient-HPLC, Casporan®, Lyophilized powder for injection.*

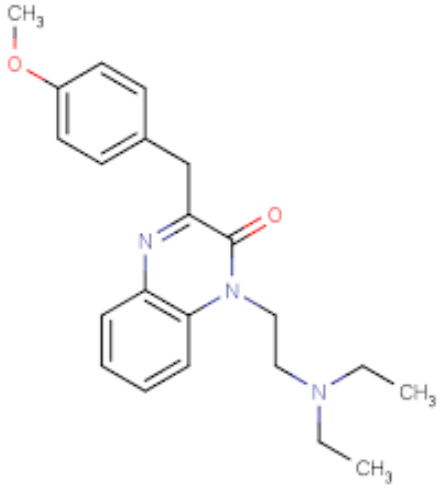
Introduction:

Caroverine 1-(2-diethylaminoethyl)-3-(p-methoxybenzyl)-1,2- dihydro-2-quinoxalin-2-on-hydrochloride is chemically derived from isoquinoline, the basic structure of papaverin. It is clinically available in some countries as a spasmolytic drug based on its unspecific Ca^{2+} channel blocking activity for more than 40 years. Caroverine is a drug used as a spasmolytic and otoneuroprotective (inner ear protective) agent in some countries. It acts as an N-type calcium channel blocker, competitive AMPA (α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid receptor) receptor antagonist, and non-competitive NMDA (N-methyl-D-aspartate receptor) receptor antagonist [1, 2]. It also has potent antioxidant effects [3]. In Pakistan, caroverine is marketed as Saprina tablets 20 mg (Biopharma, Multan, Pakistan) for oral smooth muscle spasms.

The entire inspection of literature for caroverine determination disclosed that not a single analytical method is available. Up to our knowledge the assay of caroverine in pure and dosage forms is not official in any pharmacopoeia and therefore, requires much more investigation. However we are very first time reporting here a best and latest analytical method for the determination of caroverine using a sensitive analytical techniques like RP-HPLC.

Due to non-availability of any analytical procedure for the determination of caroverine in pharmaceutical raw and dosage forms a very intensive care has been taken to develop this method. The purpose of this study is to develop a validated analytical method by RP-HPLC to quantify caroverine in pharmaceutical raw and dosage forms. The reported method was validated according to International Conference on Harmonization (ICH) guidelines [4]. The stability indicating property of the proposed method was also evaluated.

Figure-1: Chemical structures of Carvoverine



Experimental:

Chemicals and reagents: Carvoverine was obtained as kind gift sample from Hetro- Ltd, Hyderabad. Potassium dihydrogen ortho-phosphate, Acetonitrile 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 Tinnex® 0.2 mg film coated Injections, Bayer, India contains 0.2 mg of Carvoverine, 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 µL/ 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. Zorbax Eclipse XDB-C18, (150x4.6mm, 5µm particle size was used for separation of Carvoverine. 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 2.5 with dilute *ortho*-phosphoric acid (mobile phase solvent-A) and Acetonitrile (mobile phase solvent-B) in Gradient mode of separation was used to resolute the Carvoverine. 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 260 nm. The column oven temperature was maintained at 25◦C. Data acquisition, analysis, and reporting were performed by Empower2 (Waters) chromatography software.

Preparation of Solutions

Standard and stock solutions: Standard solution of the active pharmaceutical ingredient was prepared in the following manner: Transfer 40 mg of Carvoverine working standard into a 100 ml volumetric flask, dissolve and dilute with Acetonitrile and water in the ratio of water as diluent. 5 ml of the resulting solution is further diluted up to 50 ml in volumetric flask with diluents. The resulting solution contains 40 µg/mL of Carvoverine as working standard solutions. The prepared stock solutions were stored at 4 ^{0}C and protected from light.

Preparation of the Sample solution*:* Tinnex® (Carvoverine injection) is supplied as 160 mg/8 ml containing 20mg of Carvoverine per mL, for intramuscular administration. Active Ingredient: Carvoverine. 5 vials of Tinnex® (Carvoverine injection) is supplied as 160 mg/8 ml were collected, emptied their contents. Then they are uniformly blended and a quantity equivalent to 40 mg was collected and transferred in to a 100-mL volumetric flask, in mixed with diluient 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 20 µg/mL of Carvoverine as working sample solutions. The prepared stock solutions were stored at 4 ^{0}C and protected from light.

Solutions for validation study:
Calibration and Quality control samples: Calibration standards (4-48 µg/ mL of Carvoverine were prepared from working standard solutions by appropriate dilution with water in the as diluents. Quality control (QC) samples for accuracy studies were prepared at three concentrations of the linearity range (32µg/ mL, 40 µg/ mL and 48 µg/ mL) for Carvoverine 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 IM injections 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 20 µg/ mL for Carvoverine. 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 4–48 µg/ mL for Carvoverine. 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 Carvoverine

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 Intramuscualr injection 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 Carvoverine

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 (32 µg/ mL (80% dilution), 40 µg/ 2mL (100% dilution) and 48 µg/ mL (120% dilution) for Carvoverine were prepared from the standard solutions. Known amounts of 10 % dilution of drug (4 µg/mL of Carvoverine) 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 re-covered by the assay. (Table-5)

Table-5: Accuracy: recovery data for Carvoverine
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 Carvoverine 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 Carvoverine

Solution stability: To assess the solution stability, standard and test solutions were kept at 25^0Claboratory temperature) for 24 h. These solutions were compared with freshly prepared standard and test solutions.

RESULTS AND DISCUSSION:

HPLC method development: The API solution of analyte of interest i,e., Carvoverine was prepared in diluent at a concentration of 50µg/mL and scanned in UV-Visible spectrometer; and the Carvoverine was found to have UV maxima at around 260 nm. Hence detection at 260 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 Carvoverine. 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. Acetonitrile 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 Gradient 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 Gradient 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 260 nm. The injection volume was 20 µL. Acetonitrile and water in the ratio of 50:50 v/v) were used as diluent. In the optimized Gradient conditions, Carvoverine 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 Intramuscualr injection 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 Carvoverine.

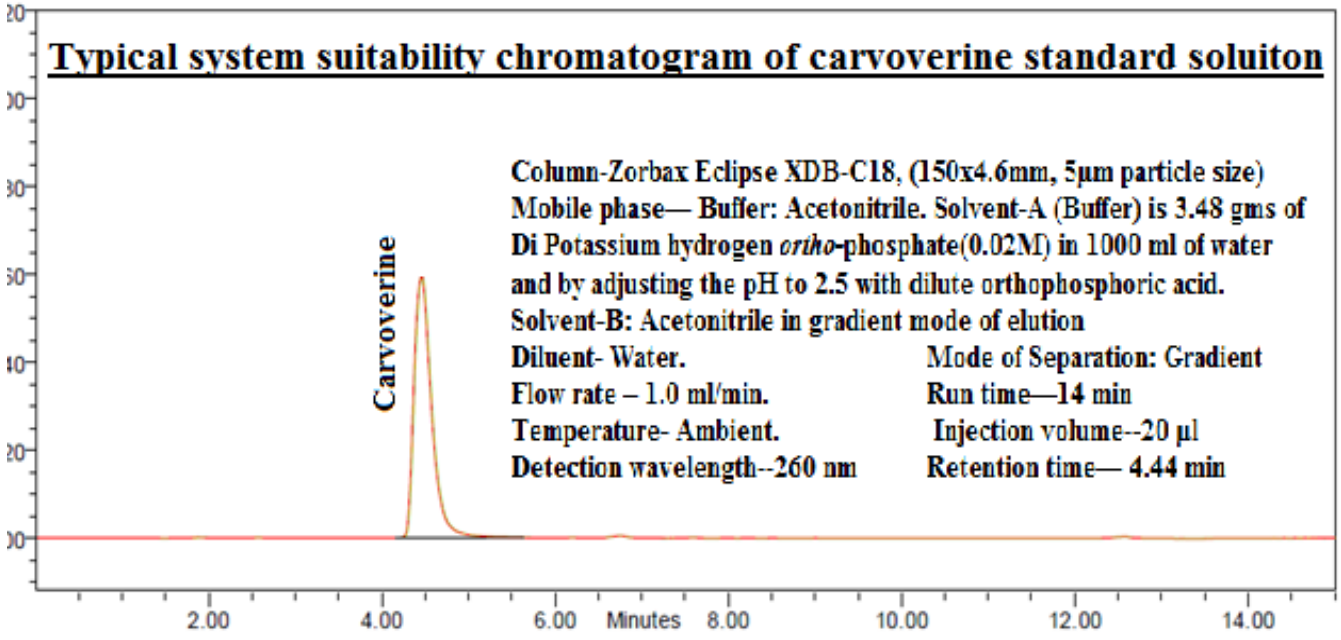


Table-2: Results of System suitability study.

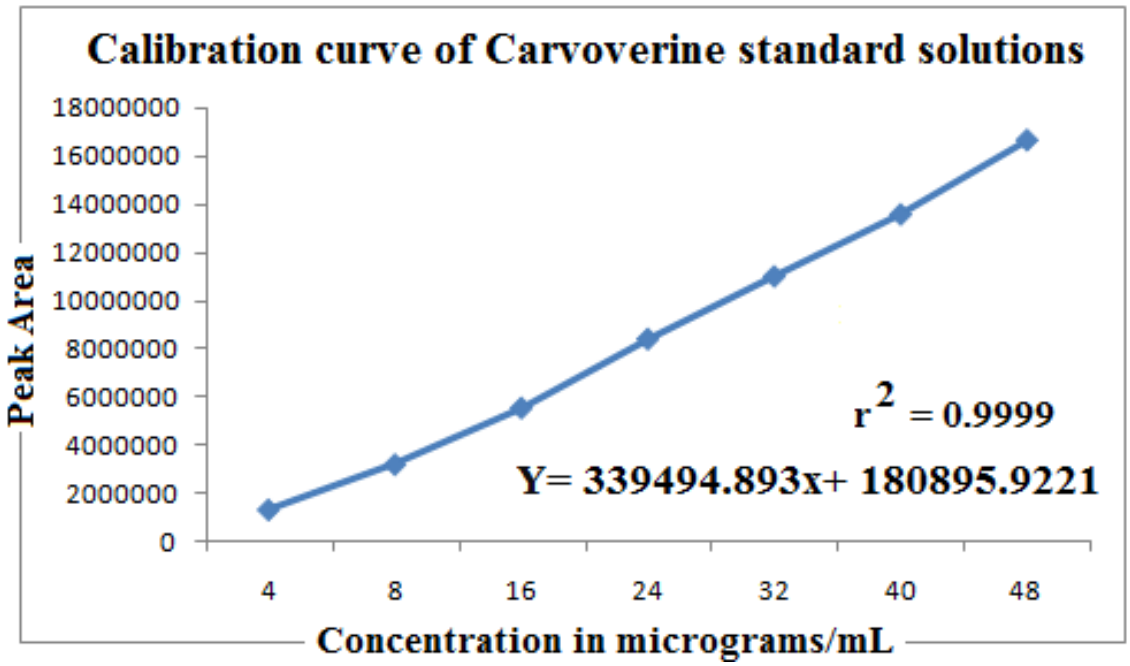
Parameter	Carvoverine
Retention time	4.45
Theoretical plates	5414.295
Tailing Factor	1.42
HETP	$2.77x10^{-5}$
USP plates/meter	36113.17423
Resolution	2.78
Peak area	
% of Peak area	99.45

***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 Carvoverine. 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 Carvoverine. The square of the correlation coefficient (r^2 > 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 Tinnex®- 0.5 mg- Film coated Injections.

Concentration	Peak Area	Parameter	Carvoverine
4 µg/ Ml	1326320	Concentration Range	4-48 µg/ mL
8 µg/ mL	3201600	Regression equation	Y= 339494.893x+ 180895.9221
16 µg/ mL	5547942	Correlation Coefficient	0.999
24 µg/ mL	8399019	0.95 Confidence interval	Lower-Limit-0.993/ Upper Limit-1
32 µg/ mL	10992339	0.95 Confidence interval	Lower-Limit-0.987/ Upper Limit-1
40 µg/ mL	13559561	Limit of Detection(LOD)	0.04 µg/ mL
48 µg/ mL	16632612	Limit of Quantification(LOQ)	0.012 µg/ mL

Figure-3: Calibration Curve of Carvoverine.



***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 Carvoverine

Precision data of Carvoverine	**Inter-day precision**		**Intra-day precision**	
Analyte-conc. (40 µg/ml)	Retention time in min.	Peak Area	Retention time in min.	Peak Area
Carvoverine injection-1	4.44	14258105	4.43	14130740
Carvoverine injection-2	4.43	14253871	4.44	14113317
Carvoverine injection-3	4.41	14244580	4.44	14085496
Carvoverine injection-4	4.43	14195650	4.46	14077114
Carvoverine injection-5	4.43	14180476	4.44	14031450
Carvoverine injection-6	4.45	14124036	4.46	14017572
Mean	4.4	14209452.9	4.4	14075948.1
Std. Deviation	0.0	52726.1	0.0	44449.8
% RSD	0.3	0.4	4.43	14130740

***Accuracy**:* Percentage recovery of the active ingredient using this method was determined using Tinnex® 160 mg/5 mL is (Carvoverine sodium Injections) is supplied as Injections containing 40 mg/mL of Carvoverine sodium, for IM 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 Carvoverine

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	11085657	12450351	13910908	15361992	17070634	18190333
2	11062914	12461202	13855482	15340595	17079835	18167831
3	11062876	12383217	13835789	15369199	17064215	18143173
Avg	11070482.3	12431590.0	13867393.0	15357262.0	17071561.3	18167112.3
Std.Dev	13141.7	42242.1	38950.2	14877.1	7851.2	23588.2
%RSD	0.1	0.3	0.3	0.1	0.0	0.1
% Recovery	102.20		112.3%		82.90	

Tinnex® sterile, lyophilized product for intravenous (IV) infusion working sample solution was spiked
-at 80% level (32 µg/ml was spiked with 10% of mixed standard solution of API's(5 µg/ml)
-at 100% level (40 µg/ml was spiked with 10% of mixed standard solution of API's(5 µg/ml)
-at 120% level (48 µg/ml was spiked with 10% of mixed standard solution of API's(5 µg/ml)

Sensitivity: Limit of detection (LOD) for Carvoverine was 0.04 µg/mL and limit of quantification (LOQ) for Carvoverine was 0.12µg/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-5: Robustness study of Tinnex® 160 mg/ 8 mL IM injection solution at 100 % level (20µg/mL):

Parameter	**Carvoverine** in Flow increase study		**Carvoverine** in Flow decrease study		**Carvoverine** in Variable column Study	
	Run time	Peak Area	Run time	Peak Area	Run time	Peak Area
Injection-1	4.05	11976336	4.78	14828243	4.42	13317464
Injection-2	4.06	11852294	4.79	14897290	4.42	13270012
Injection-3	4.05	11978216	4.80	14833574	4.41	13284434
Mean	4.1	11935615.3	4.8	14853035.6	4.4	13290636.6
% RSD	0.0	72164.4	0.0	38418.0	0.0	24326.5
Std. Dev	0.1	0.6	0.2	0.3	0.1	0.2

Analysis of the film coated Intramuscualr injection:

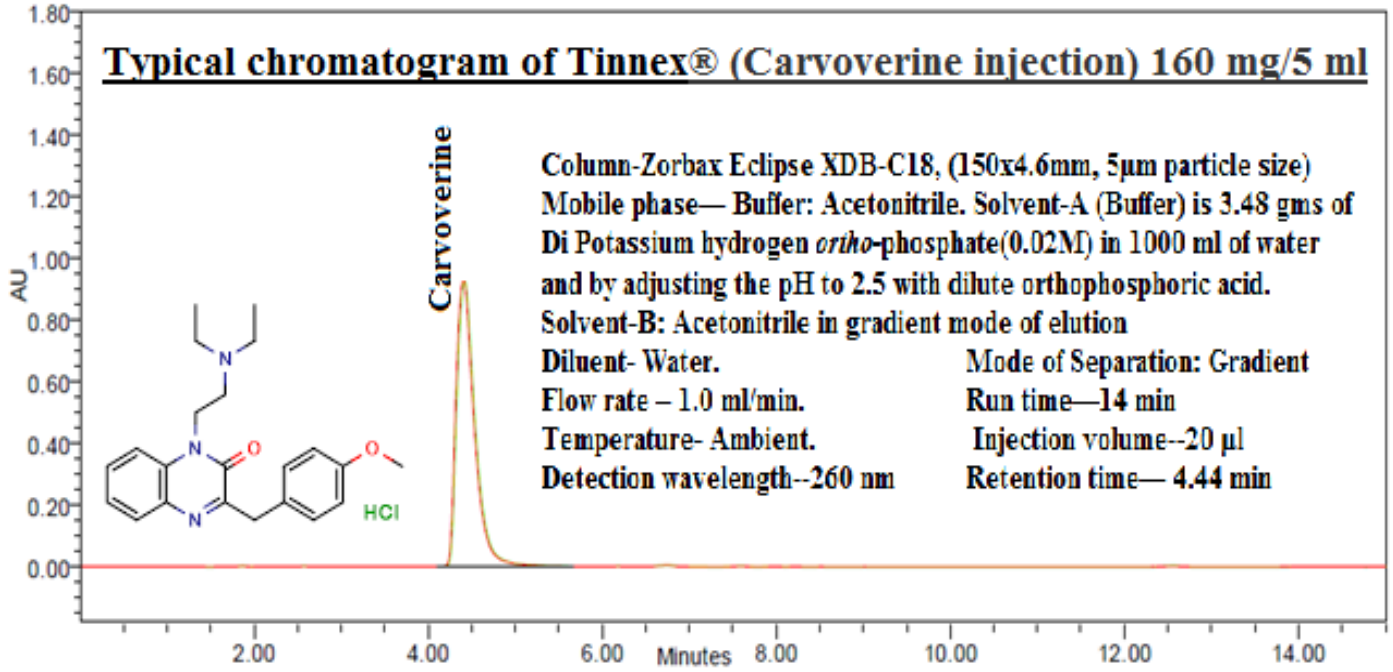
Tinnex® (Carvoverine injection) is supplied as 160 mg/5 ml containing 40mg of Carvoverine per mL, for intramuscular administration. Active Ingredient: Carvoverine. 5 vials of Tinnex® (Carvoverine injection) is supplied as 160 mg/5 ml were collected, emptied their contents. Then they are uniformly blended and a quantity equivalent to 40 mg was collected and transferred in to a 100-mL volumetric flask, in mixed with diluient by sonication, and filtered through Whatman no. 41 filter paper. 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 40 µg/mL of Carvoverine as working sample solutions. The prepared stock solutions were stored at 4 ^{0}C and protected from light. The amount of Carvoverine 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 Carvoverine present in Intramuscualr injection dosage forms. Figure-3 shows representative chromatograms obtained from the analysis of Tinnex® (Carvoverine injection) is supplied as 160 mg/5 ml. 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-6: Assay results of Tinnex®- 0.2 mg- Film coated Injections.

Formulation	Label Claim (mg/vial)	Amount found in (mg/vial)
Injection	160	156.8

Figure-4: Typical Chromatogram of Tinnex®- 0.5 mg- Injections.



Conclusion: In this study, a validated simple and reliable RP-HPLC-PDA procedure was described for the assay of a Tinnex®-160 mg/8 mL IM injections that contains a Carvoverine, 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 Injections by analytical procedure. The active pharmaceutical ingredient was successfully resolved and quantified using Zorbax Eclipse XDB-C18, (150x4.6mm,5µm particle size)) in a relatively short run time of 18 minutes in Gradient 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.

References

1. Davison M (2011) Pharmaceutical Anti-Counterfeiting: Combating the Real Danger from Fake Drugs. John Wiley & Sons, Inc.

2. Udilova N, Kozlov AV, Bieberschulte W, Frei K, Ehrenberger K, et al. (2003) Bio Pharmaco 65: 59-65.

3 Nohl H, Bieberschulte W, Dietrich B, Udilova N, Kozlov AV (2003) Caroverine, a multifunctional drug with antioxidant functions. Bio Factors 19: 79-85(1996) Q2(R1)

4. Validation of Analytical Procedures: Text and Methodology, Note for guidance on validation of analytical procedures: methodology.

