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Development of a High Performance Liquid Chromatography Method for the Determination of Related Substances in a Liposomal Nano Pharmaceutical Using Quality by Design Approach

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ABSTRACT: This study presents the development of gradient reverse phase high performance liquid chromatographic method for the determination of Doxorubicin hydrochloride and its five impurities following Quality by Design (QbD) approach and also to identify the conditions where adequate separation quality in minimal analysis duration could be achieved within a robust region that guarantees the stability of method performance. The relationship between critical process parameters and critical quality attributes is created applying Design of Experiments methodology. The defined mathematical models and central composite design are used to evaluate the risk of uncertainty in models prediction and concerns in adjusting the process parameters and to identify the design space. Moreover, Box-Behnkan design is applied for experimental robustness testing and method is partially validated to verify the adequacy of selected optimal conditions. The analytical column Waters X-bridge C18 (250 mm x 4.6 mm, 5µm particle size); mobile phase A consisted of buffer (100 mM Sodium dodecyl sulphate and 22mM of Orthophosphoric acid , pH adjusted to 2.5 with 1 M sodium hydroxide solution), acetonitrile and methanol (60:30:10) v/v/v; mobile phase B consisted of Buffer, acetonitrile and methanol (30:60:10),column temperature 40ºC, mobile phase flow rate 1 mL min^{-1}, wavelength of detection 254 nm..

Keywords: Quality by Design; Design space; Doxorubicin related substances and RP-HPLC.

INTRODUCTION: Recently trends in pharmaceutical industry and numerous regulatory documents in the pharmaceutical fields like (Food and Drug Administration's (FDA) Good Manufacturing Practice for 21st Century,[1] International Conference of Harmonization (ICH) Q8 (R2), strongly suggest the implementation of Quality by Design (QbD) concept in pharmaceutical product development and consequently, in analytical method development. QbD is defined as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management".[2] Chemometrical tools such as Design of experiments (DoE) methodology are closely related to QbD and many basic concepts are very similar.[3 &4] Therefore DoE methodology combined with methodologies for identification of design space provides deep understanding of analytical systems and enable the identification of experimental region where the quality will be assured. Since gradient reverse phase liquid chromatography (RP-LC) is the most commonly applied separation technique in pharmaceutical industry for impurities determination, the QbD concept is studied in LC systems by groups of authors such as Hubert at al.,[3- 6]Molnar at al.,[7-8] Orlandini at al.,[4, 9-10] However, the literature examination revealed that there are very less papers dealing with risk management and design space in analytical method development in recent years.[11]QbD is a dynamic approach that has gained outstanding importance in various pharmaceutical fields.[12-13] As mentioned in the International Conference of Harmonization (ICH) Guidelines Q8 (R2) for pharmaceutical development 'It is important to recognize that quality cannot be tested into the products; i.e. quality should be built in by design'. Designing the product with infusion of quality helps the product to pride over all odds during its lifecycle. As QbD is a universal approach, it has been successfully extended for the development of analytical method and can be termed as Analytical QbD (AQbD).[14-17]One of the key elements of QbD is DoE. DoE is a structured, orga-

nized method for determining the relationship between factors affecting a process and the output of that process. DoE is superior to OFAT (one factor at a time) owing to its ability to predict interactive effects of parameters on performance of the method. There are many benefits to developing analytical methods in an AQbD (Analytical quality by design) framework. Methods will be built upon the understanding of the measurement and performance requirements. The resultant method development knowledge, method robustness and transferability will result in more robust methods with fewer method failures and transfer issues The AQbD work flow starts with understanding the method needs specified in the Analytical Target Profile (ATP), followed by selection of the initial chromatographic parameters and refinement of these conditions, risk assessments to identify and DoEs to mitigate experimental risk factors[18].The end result is the robust analytical method with a well understood design space and method optima. Ideally, for selection of the initial chromatographic parameters, screening designs should be employed. The knowledge obtained from this development data was utilized to fix some of the factors at certain level and to identify the critical factors for further evaluation through DoE As mentioned earlier, DoE is of unconditional importance in QbD. Designs are available for screening variables or optimization of variables. Response surface methodology is widely used for optimization purpose. The responses obtained from the DoE experiments are statistically analyzed to provide the model relationship between the responses and the independent variables. Based on this model, the best operating conditions are determined as a part of the design space. Further, NOR (normal operating range) is identified to establish the robustness of the method. Often, Monte Carlo simulations are used as a powerful tool to derive and/or improve process capability by moving into a region of the design space which is more robust to process input variables [19-22.]

Since certain number of drugs and their impurities are polar, RP-HPLC chromatography can represent a valuable for their chromatographic separation and determination. In X-bridge columns stable chromatographic sorbent is available and are sustainable at high temperature and pH. Consequently, retention behavior and the selectivity of the chosen analytics on the selected column are very often under strong influence of the factors related to the mobile phase composition. Therefore, this fact can be considered an advantage when the aim of the research is the optimization of chromatographic separation of the analytical mixture. The incorporation of QbD strategy in related substance method development is very important therefore the aim of this study was to present the QbD method development of related substance method for the determination of mixture consisted of Doxorubicin and its related known and unknown impurities. USP monograph of Doxorubicin suggests gradient UPLC method for the quantification of Doxorubicin and its related compounds in pharmaceutical dosage forms [23]. Analytical methods are not available in literature for the determination of related substances and degradation products of Doxorubicin (fig. 1), to the best of our knowledge there is no published method for related substances Therefore, to analyze the liposomal nano pharmaceutical formulation product an analytical method needs to be developed. These methods were practiced during routine experimentation of the development laboratory batches of liposomal Nano pharmaceutical.

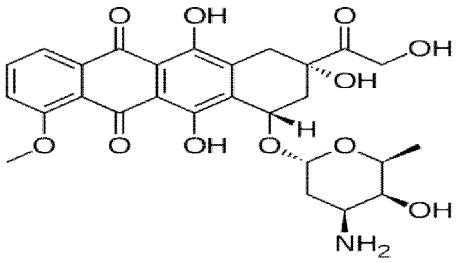

Figure 1: Structure of Doxorubicin.

MATERIALS AND METHODS: All the related impurities and Doxorubicin hydrochloride drug substance is gifted by the precise chromatography private limited. Sodium dodecyl sulphate was purchased from SIGMA-ALDRICH. Acetonitrile and Methanol were purchased from Merck. Purified water was obtained from Milli-Q water system (Millipore Corp). Sodium hydroxide and 36% Hydrochloric acid was purchased from Merck. Hydrogen peroxide (30%) was purchased from Merck. All chemicals were used as such and provided by manufacturer, no further purification has been done. All the other chemicals were of analytical grade. Magnetic stirrer, cyclomixer, micro centrifuge bought from Eppendorf Equipment's Pvt. Ltd. was used. HPLC from Waters, used in the analysis of drug. A bath sonicator from PCI Analytics brand was used.

Chromatographic system and condition: The HPLC system consists of a waters 2696 model, including quaternary pump, auto-sampler with thermostat, column oven, coupled with a multiple wavelength or diode array UV detector. Waters X-bridge C18 (4.6mm×250mm, 5µm) column for method development. The Empower chromatographic Software was

used for data acquisition and processing. Throughout the whole experimental procedure the following instrumental chromatographic conditions were maintained: flow rate of the mobile phase 1 mL min^{-1}, column temperature 40°C, UV detection at 254 nm and injection volume 20µL.

Buffer, Mobile phase and solvent mixture: Mobile phase consisted of acetonitrile, methanol and water phase (with added sodium dodecyl sulphate and orthophosphoric acid) where the amount of organic solvent, sodium dodecyl sulphate concentration in the aqueous phase and pH of the aqueous phase were varied according to the experimental plan. Mobile phase under optimal chromatographic conditions was as follows,

The mobile phase A having pH of 2.5 adjusted with 1 M sodium hydroxide solution, prepared by dissolving 100mM Sodium dodecyl sulphate and 22mM O-phosphoric acid in water, acetonitrile, methanol added in the ratio of (60:30:10) v/v/v. Similarly mobile phase B consist of buffer, acetonitrile and methanol in the ratio of (30:60:10) v/v/v.

Binary solvent gradient was applied at flow rate of 1.0 mL min^{-1} and programmed as follows: 100% mobile phase A and 0% mobile phase B at 0 min, progressing linearly at 90 % mobile phase A and 10 % mobile phase B at 20 min, followed by the decrease in the mobile phase A to 40% and rise in mobile phase B to 60 % at 40 min. Subsequently, the gradient was kept constant till 45 min, finally returning to the initial gradient and flow at 50 min and maintained at this composition and flow for 5 min in the total time of 55 min of analysis.

Standard solution mixture: Stock solutions for the method optimization and robustness testing contained 400 µg mL^{-1} of doxorubicin, and 4µg mL^{-1} of all the related impurities in the methanol. Place mixture for selectivity estimation was prepared in a concentration ratio corresponding to the content in the liposomal Nano pharmaceutical. A standard solution, containing 400 µg mL^{-1} of Doxorubicin and 4µg mL^{-1} of each related substances was utilized to prove the selectivity. Seven solutions containing Doxorubicin (100–800 µg mL^{-1}) and its related compounds (0.5–8.0µg mL^{-1}) were prepared in the methanol for linearity estimation. The accuracy estimation is performed using three series of three solutions containing placebo, Doxorubicin in concentrations 400 µg mL^{-1} and its related substances in concentrations 2.0µg mL^{-1}, 4.0µg mL^{-1} and 6.0µg mL^{-1}. The precision estimation was performed on real samples using commercially available Lipodox containing 2 mg of Doxorubicin per mL. The sample was diluted to contain 400 µg mL^{-1} of Doxorubicin and spiked with related substances in concentration of 4µg mL^{-1}. Real samples testing was performed using Lipodox diluted in methanol to obtain the working solutions theoretically containing 400 µg mL^{-1} of Doxorubicin. This procedure was repeated six times.

Control Sample solution preparation: Transfer content of vial in a dry test tube. Pipette out 2 mL of Doxorubicin Hydrochloride liposomal sample (2 mg/mL) into a clean and dry 10 mL volumetric flask, added 4 mL of methanol and sonicate for 3 minutes, further dilute to the mark with methanol.

Spike Sample solution preparation: Transfer content of vial in a dry test tube. Pipette out 2 mL of Doxorubicin HCl liposomal sample (2 mg/mL) into a clean and dry 10 mL volumetric flask, added 4 mL of methanol and sonicate for 3 minutes, further added each known impurity to the flask to obtain4µg $mL^{-1}$of impurity solution in the same flask and dilute to the mark with methanol.

Analytical target profile, critical quality attributes, risk identification, risk evaluation and statistical data analysis: First objective of this work was the thorough investigation of chromatographic behavior of analyzed substances. Selectivity is the chief motive in this case and hence the first set of CQAs on retention factor and resolution between the peaks. The second objective of the study was the development of method for the determination of Doxorubicin and its impurities where the maximal separation of substances in minimal analysis duration will be achieved. Moreover, in accordance with QbD principles, the optimal conditions should be surrounded with design space in order to provide adequate robustness of the method. As given in the USP Stimuli article the concept of an ATP parallels the concept of Quality Target Product Profile described and defined in ICH Q8. The ATP defines the objective of the analytical method and the quality requirements [24]. On the basis of scientific rationale and the development data, the factors that act maximum risk to resolution were considered as critical during risk identification. Thus, temperature of column, mobile phase organic composition (to vary the gradient slope) and buffer pH were selected as critical Variables/factors. The risk was evaluated through statistically designed experiments generated through Design expert 9.0.6.2 software (Stat Ease Inc., Minneapolis, USA). Initially, a full factorialdesign was employed, but due to the presence of curvature the design was boost to central composite design (CCD) to better understand the surface. Graphical tools such as half-normal probability plot, pareto chart

etc. were used to help assess which factor is highly important and which is comparatively unimportant. Diagnostic tools such as normal plots, residual verses predicted plots were used to evaluate the data. The right graphs, plots or visual displays of a dataset can uncover anomalies or provide insights that go beyond what most quantitative techniques are capable of discovering. Further, the relationship between the variables and the responses was estimated through regression. Analysis of variance (ANOVA) was used to assess the importance of one or more factors by comparing the response variable means at the different factor levels. The model, F-value, p-value, Lack of Fit (LoF), the R- squared values etc. revealed significance and predictability of the established model. Model graphs were used to understand the interaction between the factors and their relationship with the responses.

Design space and optimum condition establishment: Design space has been established by overlaying contours of all responses each having a predefined acceptance criterion. The objective of design space is to present a region where the method will be fit for purpose. However, since the design space is based on prediction, experimental verification was performed at random conditions within the design space to prove the agreement between the predicted and observed values. The optimum condition for each factor within the design space was identified and with the help of Monte Carlo Simulation and process capability the normal operating range was established.

Sensitivity of analysis and robustness study: Even after the establishment of design space it is necessary to demonstrate the robustness of the method given the possibility of variation in the method parameters about their standard deviation. The robustness at the optimum condition was demonstrated here by generating large amount of data with the help of Monte Carle simulation and deriving the measure of capability analysis i.e. Cpk values for responses. The analytical method robustness is strongly dependent on the sensitivity of method parameters. Hence, to find the most sensitive parameter per response, sensitivity analysis was performed using Monte Carlo simulation and the sensitivity plots were drawn using Devize software (Minitab, USA). Also, as the Cpk values are largely dependent on the standard deviation of the method variables, special attention was given to the most sensitive method variables.

The capability analysis provides a better understanding of how much variability in the output can be expected at the normal operating conditions. Finally, the optimum values and corresponding standard deviations of the factors producing data with acceptable Cpk values were utilized for the establishment of the normal operating range.

RESULTS AND DISCUSSION: This study presented the usefulness of QbD approach implementation in analytical method for related substance, the importance of this strategy in modern pharmaceutical analysis is emphasized and each step of QbD process is described in details. The definition of critical quality attributes and critical process parameters is explained. Special attention is devoted to DoE methodology application for creation of reliable mathematical models for knowledge space examination. The verification of the design space, multivariate experimental robustness testing and validation confirmed that systematic building of quality leads to the creation of highly reliable chromatographic methods.

A single, selective, and robust analytical method for the determination of related impurities in a complex liposomal Nano pharmaceutical drug product has been developed using QbD principles. About all known and unknown peaks are well separated by this method observed in force degraded samples. DoE was used to establish the relationship between the critical response and method variables. It also helped to create the design space where the global optima exist. pH plays the most critical role on the retention time of unknown impurity observed at RRT 0.96 and the resolution between two known impurity observed at RRT about 1.06. Methanol was added and gradient program was introduced to resolve the pH sensitivity issue. To establish the normal operating range, the application of Monte Carlo simulation for propagation of model uncertainty is used for creation of design space and robustness was evaluated within the normal operating range and at the edge of the design space. Finally, this method as reduced analysis time, resources, and solvent consumption in comparison also this method is cost effective compare to other related substance methods of analysis.

Identification of critical attributes and variables: The drug matrix was chromatographed on RP-HPLC system by adopting the tentative method developed through OFAT approach. The resulting chromatogram shown in (fig. 2), exhibits one API peaks, five related impurities amongst which reproducible and good resolution is desired and chromatogram shown in(fig.3), exhibit placebo peak.

The attributes from analytical target profile identified as critical are given below (fig. 4):

1. Retention time of the unknown impurities observed at relative retention time about 0.88.
2. Retention time of the related impurities observed at relative retention time about 1.19.
3. Resolution between two known impurities observed at relative retention time about 1.04.
4. Resolution between closely eluting impurities observed at the fronting of main peak Doxorubicin about relative retention time 0.96.
5. Retention time of the unknown impurity observed at relative retention time about 2.09.

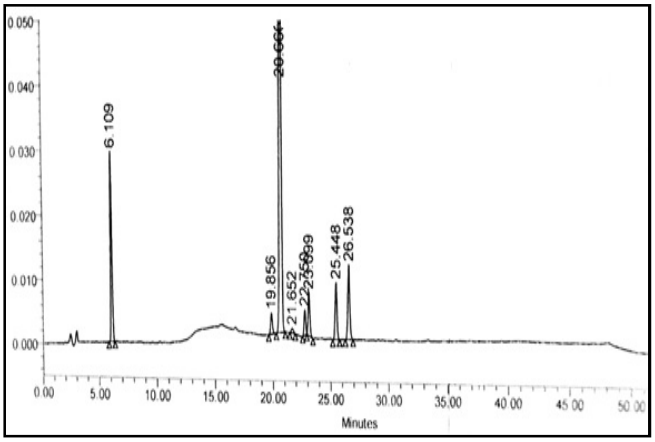

Figure 2: Chromatogram obtained though tentative method.

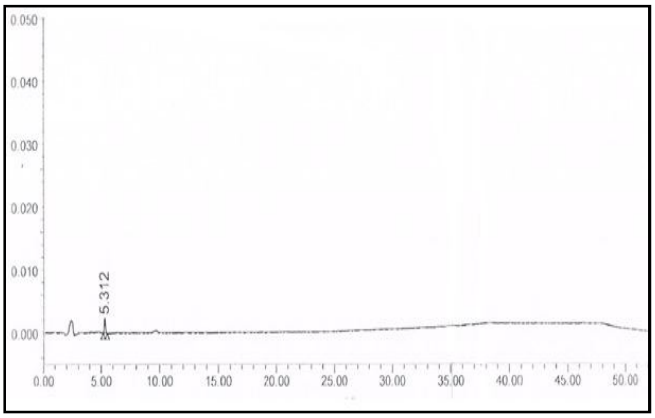

Figure 3: Chromatogram of placebo.

Of these 5 attributes, two were highly critical, the first was the retention time for unknown impurities and the second was the resolution between known impurity the retention time was found to be unstable, during random OFAT trials this impurity was observed to be co-eluting with the Doxorubicin main peak and one of the known impurity observed at RRT 0.96 and 1.08 respectively. It causes interference in the determination of the known impurity. Both the above mentioned criticalities were unresolved during the development of the method through conventional approach.

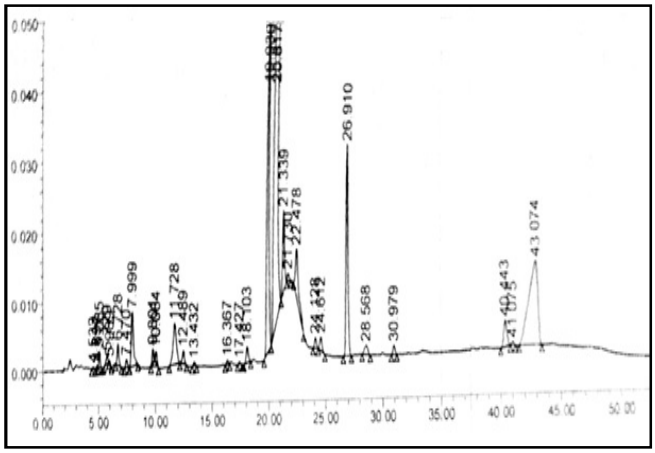

Figure 4: Trial Chromatogram before deciding analytical target profile.

Hence, the QbD approach was adopted with an intention of solving these issues and attaining a robust method with the help of DoE. Next, the listing of critical attributes was followed by identification of the variables using Fishbone diagram (fig. 5) as a risk assessment tool.

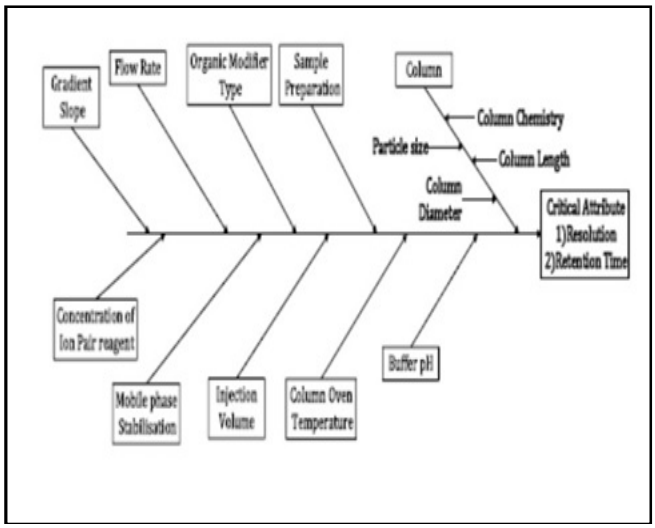

Figure 5: Fishbone Diagram for DOE trial.

Since, it is not feasible to experimentally investigate each variable; the inspection of the critical variables is essential which demands rigorous brain storming. Thus, on the basis of the development data paired with scientific rationale and previous experience, the incidental factors were neglected and the factors posing maximum risk (temperature, mobile phase composition, and buffer pH) were chosen as critical. The high and low levels for the critical factors were finalized for experimentation.

Design of Experiment: A 2^3 full factorial design was adopted to evaluate the three independent variables buffer pH, MP composition (for varying the gradient slope) and temperature. Each variable was investigated at two levels: low and high along with the center points as given in (Table 1).

Table 1: Critical factors identified and their ranges for DOE run.

Critical Factors	Low	Centre	High
Buffer pH	2.0	2.5	3.0
Temperature(°C)	25	40	60
Mobile phase composition (%)	5	10	20

Total eleven experiments were performed including eight factorial and three center points. The center point experiments illustrate curvature if present, and replicates serve in demonstrating system suitability. The runs were randomized to avoid systematic error [25].Factorial design points and the resulting dataset gives the Supplementary data. Responses were analyzed statistically by using Design expert software. The multivariate analysis proves that all the three variables studied through DoE are significant and the regression analysis provides evidence for their contribution in variation in the responses. From the main effects plots as well as from the information given in (Table 2), it can be realized that most of the responses displays curvature effects, i.e. the Centre point data is not in line with the high and the low values, hinting the existence of a non-linear relationship between the factors and the response.

Table 2: Normal operating range within the design space for robust chromatographic separation.

Factor	Optimum	Low	High
pH	2.5	2.0	3.0
Column Temperature	(40°)	(35°)	(45°)
Methanol Organic composition	10%	9%	11%
Acetonitrile organic composition	10%	27%	33%

The data indicate that pH is the most important factor in maximum number of cases followed by temperature and mobile phase composition, curvature contribution is as high as 50% in a couple of cases[Resolution between an unknown impurity and Doxorubicin peak observed at RRT 0.96 and resolution between two known Impurities observed at RRT 1.08]. Though the full factorial design offered some valuable information, incorporation of a response surface design was necessary to model the design space adequately. To serve the purpose additional axial points were studied through CCD (α = 1.47), with wider range of responses to get more information along with two additional Centre points. The complete CCD design (factorial, Centre and axial points) along with the responses is gives the Supplementary data.

Establishment of design space: As mentioned in the ICH guideline Q8 (R2) for Pharmaceutical Development, design space is the multidimensional combination and interaction of input variables. Any set point within the (regulatory approved) design space will produce acceptable product and changes within the design space are (regulatory) acceptable. The design space is the integral and conclusive part of any study through the QbD approach. But, due to the completely distant pH requirements for the two responses discussed earlier (retention time for unknown impurity about RRT 0.96 and resolution between two known impurities at RRT about 1.06) establishment of design space is challenging. An alternative approach was adopted to establish the design space by ignoring these two pH dependent responses one at a time. Exhibit the design space neglecting the retention time of unknown impurity at about RRT 0.96 and the design space neglecting the resolution two known impurities at RRT about 1.06, respectively. However, to authenticate the predictions, it's important to verify the design spaces experimentally. For the design space verification, experimentation was performed at one point in each of the design spaces. The mobile phase composition was fixed to an optimum percentage. For verification of the design space obtained by excluding resolution between two known impurities; the pH value at the Centre i.e. 2.5 was selected. Similarly, for the verification of the design space obtained by excluding retention time for unknown peak the Centre pH value i.e.2.5 was chosen. In both the conditions the column oven temperature was set at the temperature (35 ± 2°C). Thus, the two experimental conditions were i) pH: 2.0, Temperature: 35°C, % organic in mobile phase B: 30 and ii) pH: 3.0, Temperature: 35°C, % organic in mobile phase B: 30. The results indicate that all the responses are within the prediction interval (PI) and close to the 95% confidence interval (CI). The small difference between the predicted and observed values can be attributed to the fact that during the DoE experimentation, the critical factors e.g. pH were not strictly controlled and were allowed to be within their natural variability. But, during the robustness study it was observed that few responses were impacted by the small change within the natural variation of the factors. Therefore, it is quite possible that minor variations in the sensitive factors during DoE have most likely contributed to this variation. Once the method is optimized, stringent controls on variables are required as a control strategy.

CONCLUSION:

Final optimization of chromatographic condition: From the above discussion it is clearly evident that it

was not possible to achieve both the responses within the specified acceptance criteria on the same method using a single buffer pH. To resolve this issue the gradient program was modified by changing the mobile phase B and gradient from buffer, acetonitrile to buffer pH 2.5,acetonitrile and methanol, thus the mobile phase is now comprised of three mobile phase components viz. buffer pH 2.5,Acetonitrile and methanol. The gradient was reconstructed to introduce methanol at the middle so that it governed the retention of unknown peak. After a few minutes, the gradient was linear to control the resolution between known peaks observed at RRT 1.08. This finalized gradient was experimentally evaluated and slight changes were made in the factors for the better separation of the minor peaks. These changes were within the boundary of the design space. The modifications given in (Table 3) consisted of revision of optimum temperature to 40°C the modified gradient program is the optimized analytical methodology is finalized by performing validation parameters as per ICH guideline[26] mentioned in (Table 4).

Table 3: Final gradient program for the analysis of impurity profile.

Time	Flow/min	% Mobile phase A	% Mobile phase B
0	1.0	100.0	0.0
10	1.0	100.0	0.0
20	1.0	90.0	10.0
35	1.0	40.0	60.0
45	1.0	40.0	60.0
50	1.0	100.0	0.0
55	1.0	100.0	0.0

Table 4: Summary of partial validation and System suitability parameters.

Parameter (Units)	Doxorubicin	Related impurity
Linearity range (µg/ml)	100-800	0.5-8.0
Observed Correlation coefficient	0.999	0.998
LOD (µg/ml)	0.07	0.1
LOQ (µg/ml)	0.20	0.32
Range of Recovery (%)	Not applicable	92-105
System Precision (% RSD)	0.96	Not applicable
Method Precision (% RSD)	0.78	Less than 3.7
Robustness	Robust	Robust
Resolution	4.4	1.4

Sensitivity analysis study: Design space has been established and the method has been optimized, but how robust is the output response due to method input variables, pH, temperature, and mobile phase composition Even with the best controls in place, it becomes important to study the effect of process input variations on the output. It was observed that the probability of failure for retention time of unknown peak at the edge of the design space, under the experimental conditions pH 2.0, temperature 35°C, and organic composition in mobile phase B 10% is 61% and for difference in retention time of two known peak at RRT about 1.08 at pH 3.0, Temperature 35°C, and organic composition in mobile phase B 10%is 50% This makes it clear that at the edge of the design space the assurance of quality, for the retention time of unknown peak observed at RRT 0.96 and the difference in retention time of two known impurities is only 39% and 50%, respectively. Thus, robustness study and the identification of the robust region called as normal operating range within the design space becomes crucial to gain assurance in quality within the random variation of the method parameters. To assess this robustness, Monte Carlosimulation was performed at the optimum condition. Based on the sensitivity analysis the retention time of Doxorubicin impurity was found to be highly pH sensitive (fig. 6).

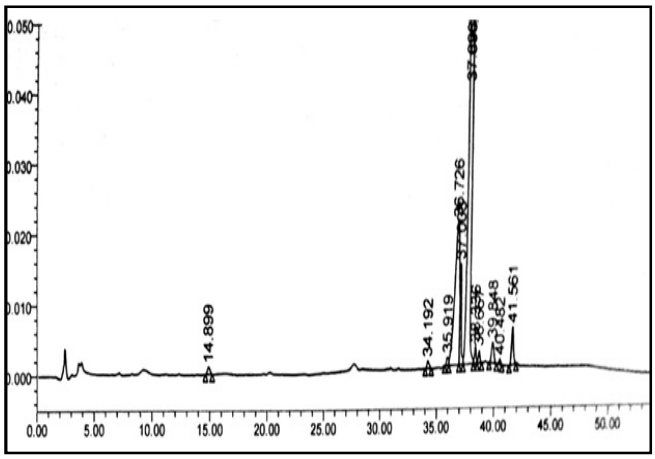

Figure 6: Chromatogram showing impact of pH on impurity profile.

Specificity: The final analytical methodology was checked with specificity, diluent chromatogram ,system suitability between Doxorubicin and Epirubicin, An alkali treated spike sample with the new gradient program is given in (Fig. 7), which clearly indicates that all the peaks are well separated and also the issues with the retention time of unknown peak observed at RRT 0.96 and the resolution between the two known impurities observed at RRT about 1.08 have been resolved in a single chromatographic run. Thus, the objective of the development of a single method for the determination of related substances for

Doxorubicin and its related impurities for the liposomal Nano-pharmaceutical has been achieved maintaining the selectivity for all peaks and thereby substantially reducing the time and resource consumption.

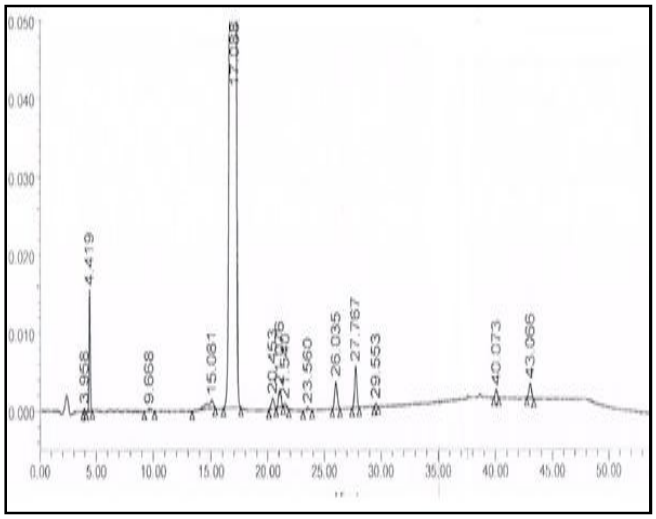

Figure 7: Impurity well separated chromatogram after alkali treatment.

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