QbD APPROACH FOR ANALYTICAL METHOD DEVELOPMENT

Quality by Design (QbD) was first outlined by Joseph M. Juran (1). It is an integrated multivariate technique which has recently gained its space in analytical method development. Quality by design is defined in ICH Q8(R1) (2) guidelines as 'a systematic approach to pharmaceutical development beginning with pre-defined objectives with an emphasis on product and process understanding and product and process control'.

The concept of QbD focuses mainly on improving robustness, quality and productivity of manufacturing processes, as well as the required quality. The development and use of analytical methods is an integrated part of the process control strategy so it is vital that a detailed understanding of the variability of critical variables in a method should be well understood. This can avoid method robustness and ruggedness issues. This thought leads to the further approach for using QbD for method development and validation (3).

Various elements of Analytical QbD (AQbD) are described as (3, 4, 5, 6):

(1) Generating the Predefined Method Objectives

The predefined objectives are called as 'Analytical Target Profile' (ATP) which are essentially used to describe the requirements of method which are essential for adequate measurement of defined critical quality attributes of the active pharmaceutical ingredient (API) or drug product. ATP defines the purpose of method which can be used for method selection, design, and development activities.

(2) Analytical Target Profile (ATP): Define target product quality profile

It is a tool that can be used in the development of any analytical method. It can be used to describe the method requirements, which are likely defined as CQAs. ATP is the combination of method performance characteristics, their criteria and CQAs, which guide the method development (7).

(3) Design and Development Phase

The Method Design Phase

The criteria defined for the ATP (or for a particular factor) allows to select a proper analytical measurement technique. After this step we need to define some starting conditions for evaluation and optimization. This can be achieved by preliminary screening of the particular factors by approaches like one factor at a time (OFAT) (traditional approach) or multifactorial Design of Experiments (DoE) approach. The DoE can explore the conditions with more thorough understanding of interdependency and interactions between method factors.

DoE is a very important quality tool of QbD. DoE is a statistical method which is used in order to find the cause-and-effect relationship between "response" (output) and factors (inputs). It is the systematic, scientific and structural way to determine the effect of critical process parameter (CPPs) [which is critical method parameters (CMPs) in analytical field] on critical quality attributes (CQAs) by creating a design space (DS). Design space is defined as "the multidimensional combination and interaction of input variables (*e.g.* material attributes) and process parameters that have been demonstrated to provide assurance of quality" (2).

Critical Quality Attributes (CQAs)

"CQAs are physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality. CQAs are generally associated with the drug substance, excipient, intermediates (in-process materials) and drug product."(The ICH guideline Q8 (R2))

- The CMPs to be optimized by DOE include various parameters like, temperature of the column oven, type of mobile phase, type of buffer, flow rate of mobile phase, composition of mobile phase, gradient time, etc.
- General CQAs to be affected by CMPs include various parameters like, resolution, capacity factor, peak asymmetry
- Various DOE tools:

  Plackett-Burman design, Taguchi design, Box-Behnken design (BBD), Central composite design (CCD), Factorial design (FD), Fractional factorial design (FFD)

- It is implemented in analytical field for purposes such as:

  To estimate the error, to avoid systematic error, to increase the precision, to widen the validity, to improve the quality and for stress degradation especially for optimization of various stress parameters like, type of acid or base, strength of acid or base, peroxide concentration (8).

(4) The Method Evaluation Phase

In this step of a structured risk assessment of the conditions laid down for the method are evaluated to identify the potential sources of variation (or the factors) affecting the method (either positively or negatively). For e.g., factors like chromatographic conditions (flow rate, organic concentration, pH, etc.) can be studied and included if found effective. A fishbone or Ishikawa diagram can be used to describe the effect of various factors (major or minor) that could influence the ATP.

Risk assessment tools like priority matrix or failure mode effects analysis (FMEA) can be used to identify how variability in a factor affects the ATP requirements. It is important to control maximum high-risk factors as possible before performing further experimentation to eliminate or minimize sources of variability. Risk assessment (9) consists of the identification, analysis and evaluation of risks that are associated with exposure to those hazards.

Risk identification: The use of information systematically to identify hazards referring to the risk question or problem description.

Risk analysis: It is the estimation of the risk associated with the identified hazards.

Risk evaluation: It compares the identified and analysed risk against given risk criteria.

ICH Q9 listed tools for risk assessment are listed below:

- Facilitation methods for basic risk management (flowcharts, check sheets etc.);
- Failure Mode Effects Analysis (FMEA)
- Failure Mode Effects and Criticality Analysis (FMECA)
- Fault Tree Analysis (FTA)
- Hazard Analysis and Critical Control Points (HACCP)
- Hazard Operability Analysis (HAZOP)
- Preliminary Hazard Analysis (PHA)

- DOE
- Risk ranking and filtering

Once the important or critical factors affecting the method have been identified and defined, the next step is to create an experimental plan through which we can find the optimum conditions for the particular factor to get desired or optimum results. The experimental plan can be defined by DoE approach which gives a systematic design inclusive of the entire range within which a particular factor shows variation. The outcome of these experiments results in the generation of the MODR (Method operable design region) i.e. the 'design space' (or proven acceptable range) across which the method meets the criteria as defined in the ATP.

(5) The Method Control Phase

The traditional approach for defining any analytical method control is through method validation and implementation of the method (application). The understanding of the method generated during the design and evaluation phases can be achieved from the QbD approach.

An ongoing program to collect and analyse the data that relates to method performance should be established across the lifecycle of the method in order to demonstrate that the ATP are continuously met. Trending such data will determine whether planned or unplanned changes affect the method impact reported results. Any changes or improvements to the method should be made with reference to the data generated to relate both, the 'Knowledge Space' (the space that covers the understanding of process parameters and input variables and their impact on critical quality factor) and MODR. Any proposed changes which take the method outside the MODR should be identifiable easily and an assessment made about whether a new MODR needs to be generated given the new criteria for the method. This new approach for improving method performance allows for advanced regulatory approaches to change the management.

A QbD approach based on a risk-assessed change control procedure which benefits from enhanced understanding of robustness and ruggedness. This has an advantage over traditional 'separate' development and validation processes in laboratories followed by repeated ICH validation exercises in the customer/receiving laboratories.

A QbD approach for analytical methods which uses risk assessment to drive robustness and ruggedness testing is more rigorous than ICH validation requirements (Q2(R1)) .

The key framework guidance documents for QbD are: ICH Q8 Pharmaceutical Development (2), ICH Q9 Quality Risk Management (9) and ICH Q10 Pharmaceutical Quality System (10). Analogous to process QbD, the outcome of AQbD (analytical QbD) is a well understood, robust and fit for purpose that consistently delivers the intended performance throughout its lifecycle.

REFERENCES

1. Juran JM. Juran on Quality by Design: The New Steps for Planning Quality into Goods and Services. USA: The Free Press; 1992.
2. ICH guidelines, validation of analytical procedure: Methodology Q8(R2). I.C.H. Harmonized Tripartite Guidelines. Geneva; 2009.
3. Hanna-Brown M, Borman P, Bale S, Szucs R, Roberts J, Jones C. Development of Chromatographic Methods Using QbD Principles. Link:http://www.sepscience.com/Sectors/Pharma/Articles/527-/Development-of-Chromatographic-Methods-Using-QbDPrinciples. Accessed on: 25 Feb 2013.
4. Yu LX. Pharmaceutical Quality by Design: product and process development understanding and control. Pharm Res. 2008; 25(4): 781-91.
5. Peraman R, Bhadraya K, Reddy YP. Analytical Quality by Design: A Tool for Regulatory Flexibility and Robust Analytics. Int J Anal Chem 2015; Article ID 868727: 1- 9.
6. Link:http://www.americanpharmaceuticalreview.com/Featured-Articles/144191-Analytical-Quality-by-Design-AQbD-in-Pharmaceutical-Development/. Accessed on: 11 June 2014.
7. Rozet E, Lebrun P, Hubert P, Debrus B, Boulanger B. Design Spaces for analytical methods. Trend Anal Chem. 2013; 42: 157-67.
8. Awotwe-Otoo D, Agarabi C, Faustino PJ, Habib MJ, Lee S, Khan MA, Shah RB. Application of quality by design elements for the development and optimization of an analytical method for protamine sulphate. J Pharm Biomed Anal. 2012; 62: 61-7.

9. ICH guidelines, validation of analytical procedure: Methodology Q9. I.C.H. Harmonized Tripartite Guidelines. Geneva; 2005.
10. ICH guidelines, validation of analytical procedure: Methodology Q10. I.C.H. Harmonized Tripartite Guidelines. Geneva; 2008.

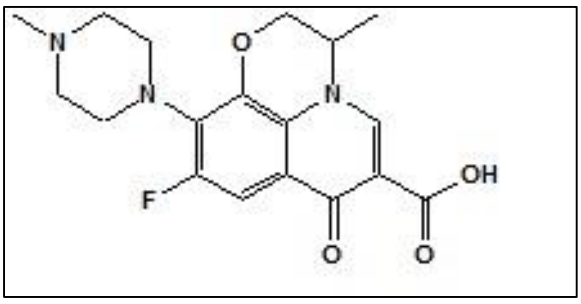
QbD APPROACH FOR DEVELOPMENT AND OPTIMISATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF FOUR COMPONENT CREAM FORMULATION: APPLICATION TO PERMEABILITY STUDY

2.1. SELECTION OF FORMULATION

The cream formulation selected for the study was a combination of four drugs i.e. Ofloxacin (OFX), Ornidazole (ORN), Terbinafine hydrochloride (TBH) and Clobetasol propionate (CBP). The cream has antibacterial (1, 2), antiprotozoal (3), corticosteroid and anti-inflammatory (4) agents having a specific activity and hence the cream has a multipurpose range of being used in various skin disorders such as atopic dermatitis and capillaris dermatitis. The four components of the cream vary widely in their pKa and log P values. The isocratic elution trials taken for the development of simultaneous RP-HPLC method were not fruitful as each drug eluted at various ratios of combination of mobile phase. Hence it was planned to apply QbD approach for the development of simultaneous RP-HPLC method development so as to minimise the RP-HPLC trials and reduce the time required for the optimisation of mobile phase.

2.2. DRUG PROFILE

(1) Ofloxacin (OFX) (1, 8)



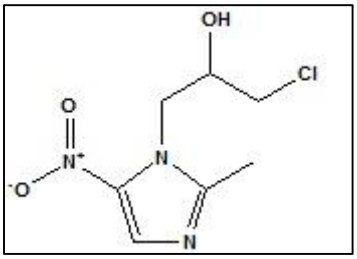
a. **Category :** Antibiotic
b. **Molecular formula :** $C_{18}H_{20}FN_3O_4$
c. **Molecular Weight :** 361.3
d. **Nomenclature:** 9-fluoro-3,7-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid

e. Physicochemical Properties:

i. Description: White to pale yellow crystalline powder
ii. Solubility: Soluble in MeOH, ACN; insoluble in water
iii. log P: 0.65
iv. pKa: 5.45, 6.2
v. Melting Point: 250-254° C

f. Official Status: Official in IP, USP, EP and BP.

(2) Ornidazole (ORN) (5, 8)



a. Category : Anti-infectant (for protozoan infections)

b. Molecular formula : $C_7H_{10}ClN_3O_3$

c. Molecular Weight : 219.63

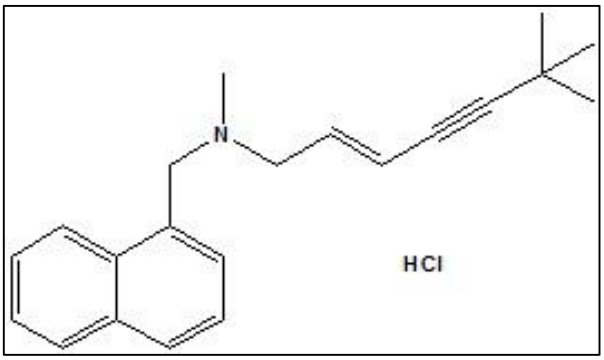
d. Nomenclature: 1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole

e. Physicochemical Properties:

i. Description: White crystalline powder
ii. Solubility: Soluble in MeOH, ACN; slightly soluble in water
iii. log P:0.69
iv. pKa: 2.4
v. Melting Point: 77-78° C

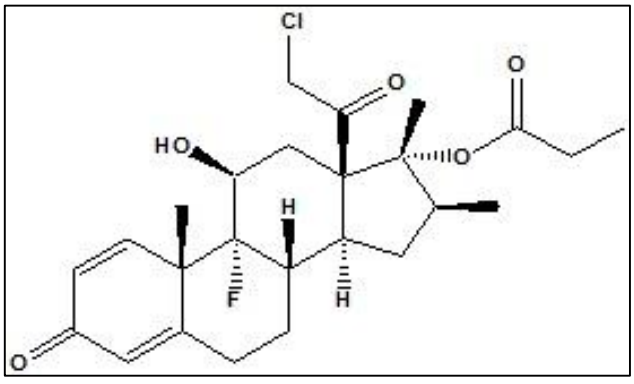
f. Official Status: Official in IP, USP and BP.

(3) Terbinafine hydrochloride (TBH) (6, 8)



a. **Category** **:** Allylamine antifungal
b. **Molecular formula :** $C_{21}H_{25}N$
c. **Molecular Weight :** 291.4
d. **Nomenclature:** (E)-N,6,6-trimethyl-N-((naphthalen-5-yl)methyl)hept-2-en-4-yn-1-amine hydrochloride
e. **Physicochemical Properties:**
    i. Description: White crystalline powder
    ii. Solubility: Soluble in MeOH, ACN; insoluble in water
    iii. log P: 6.61
    iv. pKa: 8.94
    v. Melting Point: 195-198° C
f. **Official Status:** Official in IP, USP and BP.

(4) Clobetasol propionate (CBP) (7, 8)



a. **Category** **:** Corticosteroid
b. **Molecular formula :** $C_{22}H_{28}ClFO_4$
c. **Molecular Weight :** 410.9

d. Nomenclature:21-chloro-9-fluoro-11β,17-dihydroxy-16β-methylpregna-1,4-diene-3,20-dione 17 propionate

e. Physicochemical Properties:

i. Description: White to off –white crystalline powder
ii. Solubility: Soluble in MeOH, ACN; insoluble in water
iii. log P:2.74
iv. pKa: 12.47, -3.4
v. Melting Point: 224-226° C

f. Official Status: Official in IP, USP and BP.

2.3. MARKETED FORMULATION

Various commercial formulations are available in the market for the combination under study such as: Panderm Plus cream (Macleods Pharma), Strobact Plus cream (Salius Pharma) and Zimisil Plus cream (Dewcare concept). As the formulation Panderm Plus was available in the market at the time of study so only it was taken for analysis. The cream formulations consists of 0.75%w/w OFX, 2%w/w ORN, 1%w/w TBH and 0.05%w/w CBP.

2.4. LITERATURE REVIEW

Various analytical methods have been reported in the literature for the quantitation of CBP, OFX, ORN and TBH either individually or in various combinations with other drugs using HPLC methods (9-17), UFLC method (18), stability indicating methods (19-26), HPTLC methods (27-29), UV spectrophotometric methods (30-34) and capillary electrophoresis method (35). No method was available at the time the study was undertaken though now HPLC based methods for the same combination are available in the literature (36-38). The method presented here deals with the QbD approach for the HPLC method development for simultaneous estimation of OFX, ORN, TBH and CBP. The present study deals with the study of different factors influencing the RP-HPLC method development for the simultaneous estimation. This approach explores entire risk factors for development of HPLC method and the robustness boundaries of the method. The method involves cumulative study of factors which cannot be studied in OFAT screening. The method thus optimised was applied for the assay of cream formulation and *invitro* permeability study.

2.5. EXPERIMENTAL

2.5.1. Instrumentation

Chromatography was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AD pump (binary) and Shimadzu PDA-M20A Diode Array Detector. Samples were injected through a Rheodyne 7725 injector valve with fixed loop at 20 µL. Data acquisition and integration were performed using LC Solution software (Shimadzu Corporation, Kyoto, Japan). Separation and quantitation were made on a Phenomenex C18 column (5µm × 250mm × 4.6mm i.d.). The experimental design model was developed on Design Expert software® 7.1.

2.5.2. Materials and reagents

The API of Ofloxacin and Ornidazole were provided as gift samples from Intas Pharmaceuticals, Terbinafine hydrochloride was purchased from Symbolic Pharma and Clobetasol propionate was provided by Sumit Laboratories. HPLC grade (Spectrochem) MeOH and ACN were used for the analysis. AR grade potassium dihydrogen phosphate (LobaChem), AR grade phosphoric acid (LobaChem) and HPLC grade triethylamine (Spectrochem) were used for preparation of buffer.

2.5.3. Experimental conditions

Phosphate buffer (0.02 M) was prepared by dissolving 2.72 g of anhydrous potassium orthophosphate (KH_2PO_4) in 1 L of previously filtered double distilled water, 0.05% triethylamine was added and the pH was adjusted to 2.6 using phosphoric acid. The gradient elution was carried out with the mobile phase comprising of 0.02M phosphate buffer (pH 2.6) as solvent A and ACN as solvent B. All determinations were performed at ambient temperature and at the wavelength of 258 nm. The flow rate was 1 mL/min. The injection volume was 20 µL.

2.5.4. Preparation of standard solutions

The diluent used for the preparation of all the solutions was mixture of MeOH, ACN and 20 mM phosphate buffer (pH 2.6) in the ratio of 50:25:25. All the solutions were prepared in amber coloured volumetric flasks.

OFX stock solution (2.5 mg/mL): Accurately weighed 62.5 mg OFX was transferred into 25 mL volumetric flask, dissolved and then diluted with the diluent up to the mark.

ORN stock solution (5 mg/mL): Accurately weighed 125 mg ORN was transferred into 25 mL volumetric flask, dissolved and then diluted with the diluent up to the mark.

TBH stock solution (5 mg/mL): Accurately weighed 125 mg TBH was transferred into 25 mL volumetric flask, dissolved and then diluted with the diluent up to the mark.

CBP stock solution (1 mg/mL): Accurately weighed 25 mg CBP was transferred into 25 mL volumetric flask, dissolved and then diluted with the diluent up to the mark.

From the stock solutions, appropriate aliquots were taken to prepare solution mixtures of OFX, ORN, TBH and CBP in the range of 150-750 µg/mL OFX, 400-2000 µg/ml ORN, 200-1000 µg/mL TBH and 10-50 µg/mL CBP in the mobile phase [solvent A: solvent B (50:50)], which were analysed by HPLC method under above mentioned chromatographic conditions. Chromatographic peaks were recorded at 258 nm using PDA detector.

2.5.5. Experimental Design

The DoE plan for the optimization of variables which affect the performance of developed method was based on a 3^3 full factorial design. The three key factors were: pH, initial percentage of organic (%BI) and gradient time (Tg). The design formed a chromatographic database, which was used to study the factors and enabled the selection of optimised conditions in order to get the best optimised RP-HPLC conditions. The three parameters (or factors)and their levels are shown in Table 2.1 and accordingly a set of total 29 experiments was performed including two centre points. The responses (output) selected on the basis of performance were: TF1 (tailing factor of OFX), TF2 (tailing factor of ORN), TF3 (tailing factor of TBH), TF4 (tailing factor of CBP) and RS3 (resolution between pair of peaks i.e. TBH and CBP). The resolution between other pairs of drugs was satisfied during the trials and hence only TBH and CBP resolution were taken into consideration.

Table 2.1 Factors and their levels for experimental design

Factors	Factor ID	low	middle	high
pH	A	2.5	4	5.5
%BI	B	15	20	25
Tg (min)	C	2	4	6

2.5.6. Validation of the DoE model

To validate the DoE model, six check points with various values of pH (2.8, 3, 3.5, 4.5, 4.8, 5), %BI (16, 17, 18, 19, 20) and Tg (3, 4, 4.5, 5, 6) were selected, the trials were performed and the results of the experimental values obtained were compared with the predicted values.

2.5.7. Validation of RP-HPLC method

The validation of the HPLC method was carried out in accordance with the ICH guidelines (39). The method was validated for various parameters like linearity, accuracy, precision, limit of detection, limit of quantification, sensitivity, selectivity and robustness.

The linearity of the HPLC detector response for determination of OFX, ORN, TBH and CBP was evaluated by analysing a series of different concentrations of each compound. The calibration range was established with respect to the practical range (according to content and ratio of each compound in the cream formulation) necessary, to give accurate, precise and linear results. Seven concentrations were chosen, ranging from 150-750 µg/mL OFX, 400-2000 µg/mL ORN, 200-1000 µg/mL TBH and 10-50 µg/mL CBP and the linearity was determined.

For evaluation of the precision estimates, inter-day and intra-day precision were performed at three concentration levels in triplicates. The three levels were: 150, 300, 450 µg/mL for OFX; 400, 800, 1200 µg/mL for ORN; 200, 400, 600 µg/mL for TBH and 10, 20, 30 µg/mL for CBP. The peak areas of all four drugs were calculated for each trial. The experiment was repeated three times in a day for intra-day precision and on three different days for inter-day precision.

Accuracy was determined by standard addition method at three levels of standard addition *i.e.* 80%, 100%, and 120%. The final concentrations at levels 0, 80, 100 and 120% were: 150, 270, 300, 330 µg/mL for OFX, 400, 720, 800, 880 µg/mL for ORN; 200, 360, 400, 440 µg/mL for TBH and 10, 18, 20, 22 µg/mL for CBP. The resulting mixtures were analysed (in triplicates) and results obtained were compared with the expected results.

According to ICH recommendations (39), the approach based on the standard deviation (S.D.) of the y-intercept and the slope was used for determining the limit of detection (LOD) and limit of quantitation (LOQ).

Various factors were assessed to check the robustness of the method. The factors such as: pH (2.4, 2.6, 2.8), Tg (3, 4, 5 min) and %BI (23, 24, 25 %v/v) were varied in the region of design space, generated by applying QbD in method optimization. The method was also found to be robust for the flow rate change of ±0.1 mL/min.

The standard stock solutions prepared in the mobile phase exhibited no chromatographic or absorbance changes for 24 h when kept at room temperature and for 48 h when stored in refrigerator (8-25 ºC).

According to ICH recommendation, the method is specific when the results are unaffected by the presence of the dosage form excipients. The specificity of the method was assessed by the formulation analysis to check interference of any excipients.

System suitability parameters such as theoretical plates, symmetry factor and resolution for OFX, ORN, TBH and CBP were calculated for n=6 replicates to study the system suitability of HPLC method.

2.5.8. Analysis of cream formulation

500 mg of cream was taken in 10 mL of mixture of MeOH, ACN and phosphate buffer (pH 2.6) in a ratio of 50:25:25 respectively. The mixture was shaken vigorously, sonicated for 10 min and was filtered through 0.2 µ membrane filter. From this mixture 4 mL aliquot was taken and diluted to 10 mL with mobile phase i.e. ACN and phosphate buffer of pH 2.6 (50:50 ratio) and injected into the HPLC.

2.5.9. Permeability study

Permeability study was carried out using Franzch diffusion cell for time period of 24 hrs. The receptor media used for the study was mixture of PBS (pH 7.4): ethanol 70:30. The receptor media was kept at a constant temperature of 37°C and stirred using magnetic stirrer. The study was carried out on the rat skin mounted on the diffusion cell. The skin was allowed to stabilise with the receptor media for 30 min and 1 gm of cream was loaded into the cell, 5 mL aliquot was withdrawn from the receptor media and replaced by an equal volume of fresh receptor medium, at appropriate time intervals (1, 2, 3, 4, 5, 6, 7, 22 and 24 hours). The aliquot was diluted upto 10 mL with the mobile phase, filtered by 0.2µ membrane filter and analysed by HPLC.

2.6. RESULTS AND DISCUSSION

2.6.1. Screening of factors

QbD based DoE was followed for optimisation of chromatographic conditions. Preliminary screening of several factors such as different columns (RPC8, RPC18), temperature (25º C, 40º C), various buffers (phosphate buffer, ammonium acetate buffer), concentration of buffer (20mM, 30mM, 40mM), organic solvent, etc. was carried out to find out main factors affecting the HPLC separation of all the four drugs. The preliminary screening also gave the ranges within which various factors required optimisation.

The Phenomenex RPC18 column was found to give good elution as compared to RPC8 as TBH and CBP did not had symmetric peak shapes and CBP had long elution time. Temperature did not have a profound effect so it was kept ambient. Various buffers such as phosphate buffer, ammonium acetate buffer and ammonium formate buffer were tried out of which symmetric and sharp peak shapes were obtained in phosphate buffer. The other two had problems such as very high elution time of TBH, high tailing of OFX and asymmetric peak shape with ORN. Hence phosphate buffer was selected as aqueous medium for the method.

The pKa values of all four drugs i.e. OFX (5.45, 6.2), ORN (2.4), TBH (8.94) and CBP (-3.4, 12.47) cover a wide range hence it was quite difficult to decide a proper

pH of the mobile phase. However at pH greater than 5.5 the retention time of TBH increased upto more than 30 min and hence the pH range of 2.5-5.5 was considered as a prime factor for study. The buffer strength from 20mM to 50mM were tried but showed no considerable effect on the SST parameters of the four drugs; hence 20 mM was selected owing to easy miscibility of the organic phase (ACN) with the buffer.

The organic solvents screened were MeOH, ACN and mixture of MeOH and ACN, but good results were obtained with ACN alone as compared to other two, as ACN gave desired peak shape and shorter run time. The four drugs eluted at different ratios of mobile phase and hence a gradient method had to be set up. The initial organic concentration (%BI) range of 15-25% and the gradient time *i.e.* the time required to change the organic concentration from %BI to 70%, these two factors were found to be critical in setting the gradient. Thus the two factors i.e. initial percentage of organic phase in the gradient and the gradient time were prime factors in optimising the gradient programme. Hence it was thought to apply QbD to study the effects of three specific factors such as pH, %BI and Tg in a systematic way and to optimise their values in such a way to obtain an HPLC method which is best suitable for the simultaneous estimation of the four drugs under study.

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. The overlay zero order spectra for all four drugs have been shown in Figure 2.1. From the UV spectrum, 258 nm was chosen as the optimum wavelength.

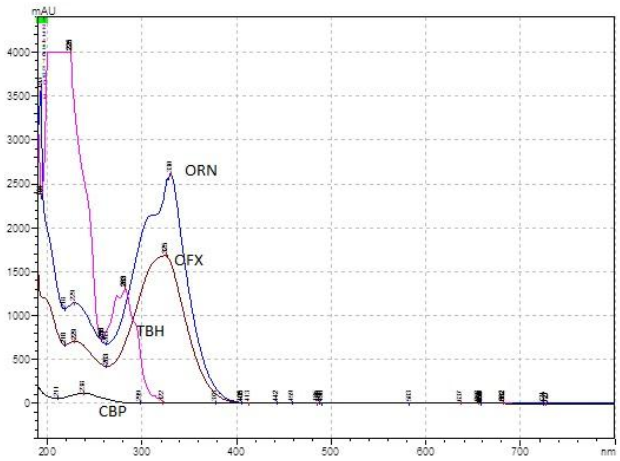


Figure 2.1 Zero order spectra of all the four drugs for selection of optimum wavelength

2.6.2. The design of experiments

The full factorial design including 27 runs (and 2 centre points) was worked out using three factors: (i) pH of buffer (2.5-5.5), (ii) %BI of ACN (15-25) and (iii) gradient time, Tg (2-6). Few trials have been shown in Figure 2.2. The model summary statistics and results of 29 runs are shown in Table 2.2. In analysis window the model were chosen on the basis of ANOVA results which showed significant p-value, R^2 value and F value. It also showed reasonably good agreement between adjusted and predicted R^2 value. The model equations of various responses have been shown in Table 2.3 and the ANOVA results along with the statistics are given in Table 2.4. The contour (2D) plots of responses with respect to all factors are shown in Figure 2.3, 2.4, 2.5, 2.6 and 2.7 for TF1, TF2, TF3, TF4 and RS3 respectively. The optimum conditions were calculated using numerical optimization. To achieve the composite desirability (D), the response criteria were set as (lower–upper): TF1 as (0.5-1.8), TF2 as (0.5-1.8), TF3 as (0.5-1.8), TF4 as (0.5-1.8) and RS3 as (>2). The Derringer's desirability was calculated for the set criterions, which indicated that maximum desirability was achieved at 0.84.

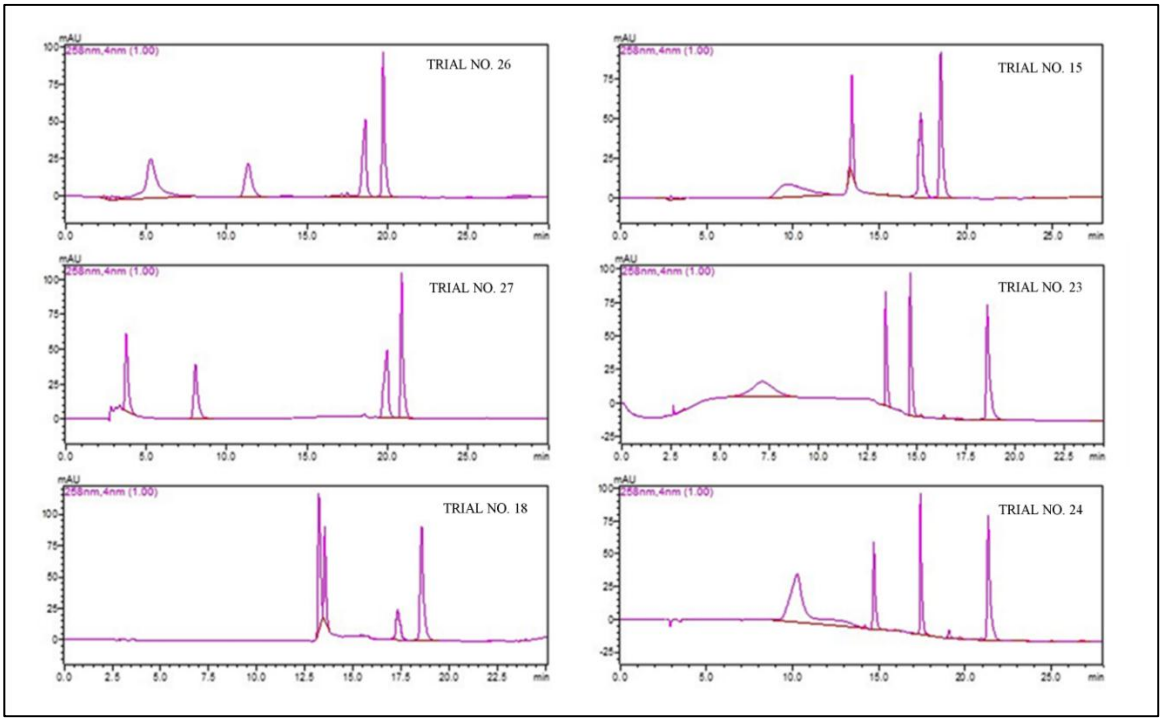


Figure 2.2 Chromatograms of few QbD trials

(1st peak OFX, 2nd peak ORN, 3rd peak TBH, 4th peak CBP)

Table 2.2 QbD trials and responses

Trial No.	Factors			Responses				
	pH	%BI	Tg	TF1	TF2	TF3	TF4	RS3
1	5.5	15	6	1.87	1.44	1.49	0.88	2.15
2	2.5	20	4	0.91	1.29	2.52	1.97	10.89
3	2.5	25	4	1.40	1.38	2.51	1.79	13.32
4	4	15	6	1.13	1.65	0.82	2.02	0.85
5	4	20	4	1.06	1.22	0.82	1.72	6.96
6	2.5	20	2	1.20	1.16	2.45	1.85	10.23
7	4	15	2	1.04	2.57	0.70	2.27	1.54
8	2.5	15	2	2.57	2.35	2.56	2.17	0.95
9	4	20	2	1.53	1.22	0.84	1.66	7.82
10	5.5	20	2	1.67	1.45	1.52	0.92	0.85
11	2.5	20	6	0.89	1.27	2.60	1.87	10.48
12	2.5	25	6	1.43	1.35	2.57	1.70	13.05
13	4	25	4	1.81	1.43	0.85	1.78	10.82
14	5.5	20	6	3.88	1.21	1.43	0.96	4.08
15	5.5	20	4	4.02	1.21	1.47	0.97	4.24
16	5.5	25	2	4.57	1.30	1.16	1.33	4.76
17	4	25	6	1.84	1.49	1.80	1.73	11.49
18	5.5	15	4	1.75	1.50	1.52	0.89	1.97
19	4	15	4	1.11	2.00	0.84	2.11	1.14
20	5.5	15	2	1.47	1.84	1.52	1.04	1.27
21	2.5	15	6	2.31	1.56	2.57	2.14	2.51
22	2.5	15	4	2.27	1.95	2.34	2.38	2.50
23	4	20	6	1.58	1.24	0.81	1.73	7.92
24	5.5	25	4	4.36	1.29	1.11	1.29	4.77
25	5.5	25	6	4.32	1.31	1.30	0.91	4.97
26	4	20	4	1.53	1.22	0.84	1.66	7.82
27	2.5	25	2	1.52	1.37	1.30	1.84	13.34
28	4	25	2	1.89	1.39	0.84	1.79	9.95
29	4	20	4	1.33	1.30	0.83	1.81	8.63

Table 2.3 Model Equations (in terms of coded values)

Responses	Equations
TF1	$TF1 = 1.325 + 0.744A + 0.423B + 0.101C + 0.913AB + 0.251AC - 0.053BC + 0.893A^2 + 0.269B^2 - 0.0616C^2$
TF2	$TF2 = 1.294 - 0.064A - 0.253B - 0.119C + 0.073AB + 0.006AC + 0.183BC - 0.117A^2 + 0.375B^2 + 0.0434C^2$
TF3	$TF3 = 0.919 - 0.496A - 0.0513B + 0.135C + 0.009AB - 0.117AC + 0.189BC + 0.973A^2 - 0.050B^2 + 0.040C^2$
TF4	$TF4 = 1.778 - 0.474A - 0.098B - 0.053C + 0.174AB - 0.032AC - 0.016BC - 0.364A^2 + 0.164B^2 - 0.044\ C^2$
RS3	$RS3 = 7.854 - 2.679A + 3.977B + 0.366C - 2.054AB + 0.232AC - 0.024BC - 0.696A^2 - 1.524B^2 - 0.352C^2$

A, B and C are the coded values for the factors pH, %BI and Tg

The model equations are of quadratic order. The factors in the equations are coded as "A" stands for pH; "B" for %BI and "C" for Tg. The coefficients of the coded values (A, B, C) describe the influence of the factors on particular response. For e.g. in first equation (for TF1): TF1 is positively influenced by A, B and C. The terms AB, BC and AC show the correlation between the two factors and their combined effect on the particular response. The increase in the values of the three factors (A, B, C) would increase TF1 response, but considering the combined effect of AB, BC and CA the combined effect of BC influences TF1 negatively hence B and C factors need to be optimised for TF1. The terms A^2, B^2 and C^2 show the quadratic curve effect for the response TF1. The positive impact of A^2 on TF1 indicates that increasing factor A will increase TF1 upto a certain level beyond which it would decrease. The magnitude of A^2 (0.893) is more than A(0.744) hence more emphasis should be given on the A^2 term for understanding the behaviour of factor A on TF1. Similarly the various equations have their significance on the basis of the coefficients of respective terms in the equation.

Table 2.4 Model Summary statistics

Statistical parameters	Responses				
	TF1	**TF2**	**TF3**	**TF4**	**RS3**
SD	0.47	0.15	0.28	0.11	1.15
Mean	2.01	1.48	1.52	1.63	6.26
CV%	23.40	9.89	18.28	6.98	18.40
R-Squared	0.88	0.88	0.89	0.96	0.95
Adjusted R^2	0.82	0.82	0.84	0.94	0.93
Predicted R^2	0.71	0.7	0.69	0.89	0.88
F value	15.3	15.3	17.15	48.71	41.13
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

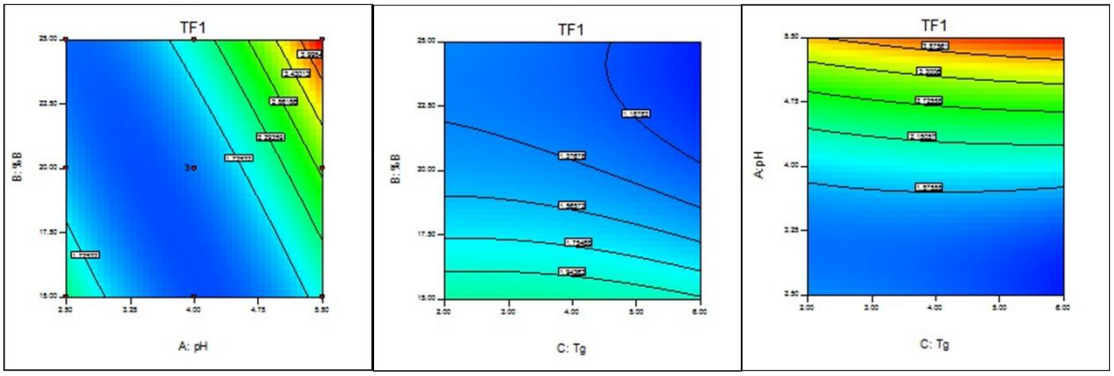


Figure 2.3 2D contour plots of response TF1 with respect to all the three factors

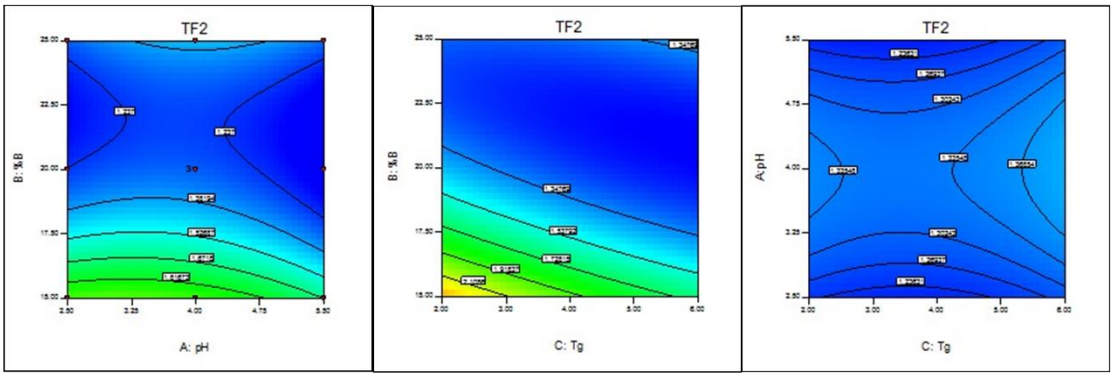


Figure 2.4 2D contour plots of response TF2 with respect to all the three factors

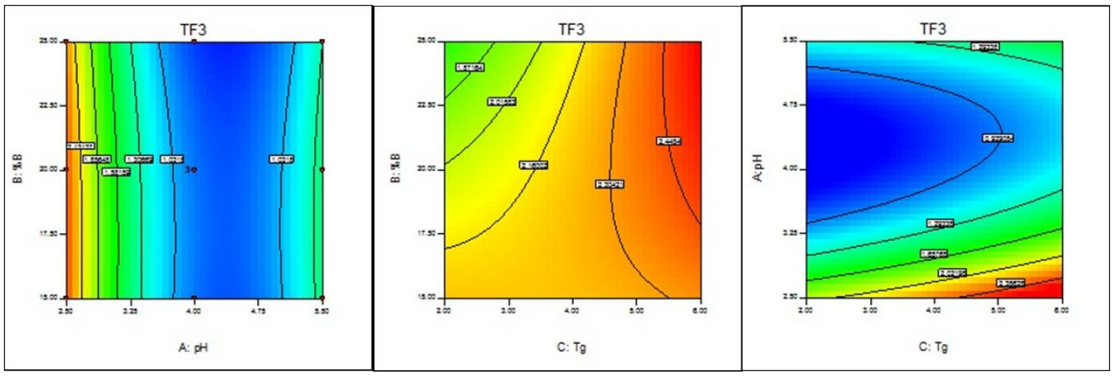


Figure 2.5 2D contour plots of response TF3 with respect to all the three factors

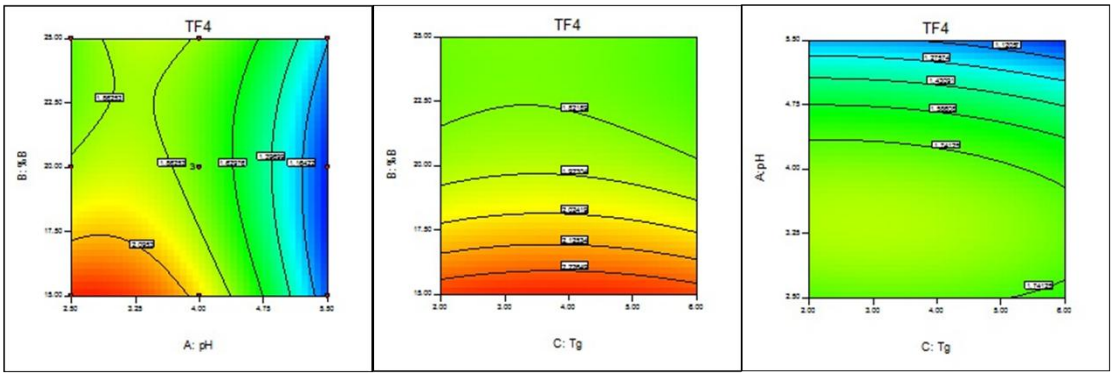


Figure 2.6 2D contour plots of response TF4 with respect to all the three factors

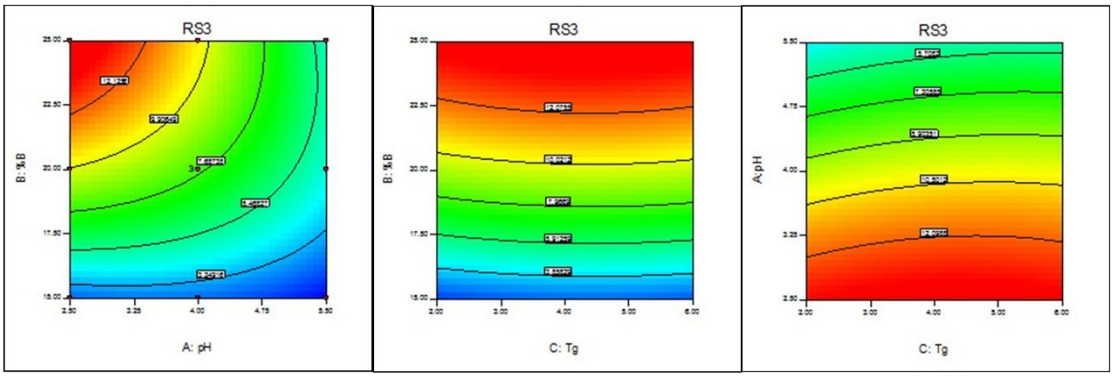


Figure 2.7 2D contour plots of response RS3 with respect to all the three factors

2.6.3. Robustness of the DoE model

To check the robustness of the model, four solutions among the generated solutions (8 solutions) were selected and analysed. To check the point prediction the experimental values were compared with the predicted values of responses. It was found that the experimental values lie within 95% confidence and predicted intervals (shown in Table 2.5). Finally solution with pH 2.6, %BI as 24 and Tg as 4 min, was chosen to record the chromatogram which allowed the complete separation of all the four compounds under study (as shown in the chromatogram i.e. Figure 2.8).

Table 2.5 Factors and targeted criteria used in Design expert

Sol[n]	pH	%BI	Tg	Response	Pred. value	Exp. value	95% CI Predicted		95% PI Predicted	
							Low	high	low	high
1*	2.6	24	4	TF1	1.35	0.85	0.72	1.98	0.18	2.52
				TF2	1.29	0.97	1.1	1.49	0.93	1.66
				TF3	1.62	1.43	1.25	2	0.93	2.31
				TF4	1.82	1.65	1.67	1.97	1.54	2.1
				RS3	13.3	12.9	11.75	14.84	10.44	16.16
2	2.7	25	4	TF1	1.34	0.83	0.73	1.95	0.18	2.5
				TF2	1.29	1.26	1.1	1.48	0.93	1.65
				TF3	1.6	1.48	1.24	1.96	0.92	2.28
				TF4	1.82	1.74	1.67	1.97	1.54	2.1
				RS3	13.14	11.98	11.64	14.64	10.3	15.98
3	2.6	25	4	TF1	1.36	1.31	0.72	1.99	0.18	2.53
				TF2	1.3	1.25	1.1	1.5	0.93	1.66
				TF3	1.6	1.57	1.23	1.98	0.91	2.29
				TF4	1.82	1.75	1.67	1.98	1.54	2.11
				RS3	13.34	12.87	11.78	14.91	10.47	16.22
4	2.7	24	4	TF1	1.33	0.97	0.74	1.92	0.18	2.48
				TF2	1.28	1.21	1.09	1.46	0.92	1.63
				TF3	1.64	1.59	1.29	1.98	0.96	2.31
				TF4	1.82	1.75	1.67	1.96	1.54	2.09
				RS3	12.9	11.76	11.46	14.34	10.09	15.71

*Finally selected for chromatographic separation; Pred.=Predicted, Exp.=Experimental; Sol[n]=Solution

The correlation was better for solution 3 but experimental values were better for solution 1: e.g. TF1 for solution 1 is 0.85 whereas for solution 3 it is 1.31, etc. Hence for better SST solution 1 was selected.

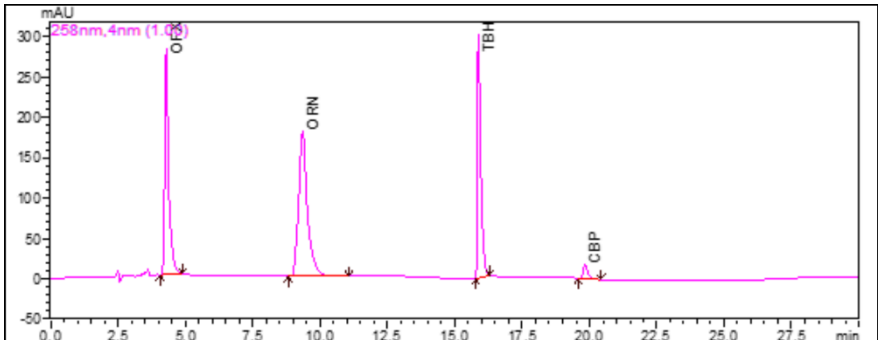


Figure 2.8 Optimised chromatogram of standard solution mixture containing 150 ppm of OFX, 400 ppm of ORN, 10 ppm of CBP and 200 ppm of TBH

2.6.4. Model validation

To validate the model, six check points were selected and the results of the experimental values obtained were compared with the predicted values. The results showed good agreement between the experimental and model generated values resulting in low residual values presented in terms of percentage bias (%bias) (Table 2.6). Figure 2.9 shows the 3D desirability contour plots and Figure 2.10 shows the design space for all the parameters. The final optimised gradient elution programme is shown in Table 2.7. Table 2.8 shows the final optimised RP-HPLC parameters.

Table 2.6 Results for validation of model (check point trials)

Trial No.	Factors			Responses	Experimental Value	Predicted value	% Bias
	pH	%BI	Tg				
1	3	20	3	TF1	1.15	1.24	0.08
				TF2	1.24	1.36	0.08
				TF3	1.56	1.59	0.01
				TF4	1.83	1.94	0.05
				RS3	9.43	9.14	-0.03
2	5	18	4.5	TF1	1.74	1.92	0.09
				TF2	1.05	1.30	0.19
				TF3	0.83	1.03	0.19
				TF4	0.75	1.30	0.42
				RS3	3.83	4.58	0.16

				TF1	1.16	1.17	0.01
				TF2	1.35	1.47	0.08
3	3.5	18	4	TF3	1.14	1.21	0.06
				TF4	1.79	1.98	0.10
				RS3	6.42	6.56	0.02
				TF1	1.23	1.43	0.14
				TF2	1.36	1.46	0.07
4	4.5	16	6	TF3	0.94	0.85	-0.10
				TF4	1.53	1.62	0.06
				RS3	3.12	3.38	0.08
				TF1	1.54	1.64	0.06
				TF2	1.24	1.39	0.11
5	4.8	17	5	TF3	0.83	0.93	0.10
				TF4	1.32	1.44	0.08
				RS3	3.91	4.11	0.05
				TF1	1.22	1.31	0.07
				TF2	1.19	1.28	0.07
6	2.8	19	5	TF3	2.18	2.05	-0.06
				TF4	2.04	1.96	-0.04
				RS3	8.44	8.37	-0.01

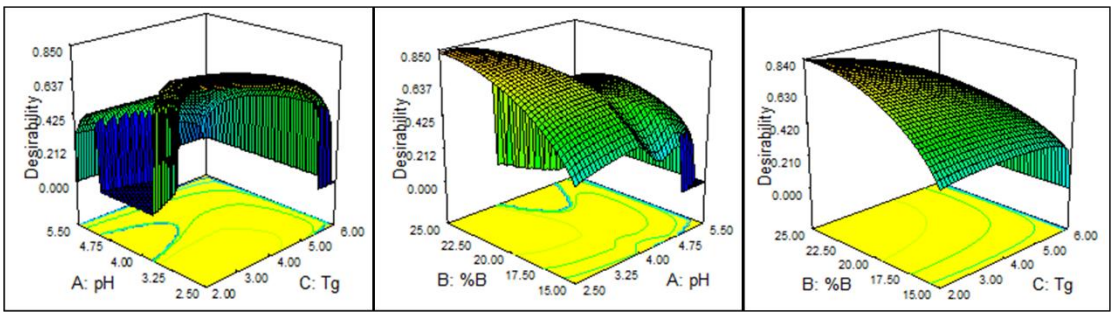


Figure 2.9 Desirability 3D plots for the three factors for optimised method

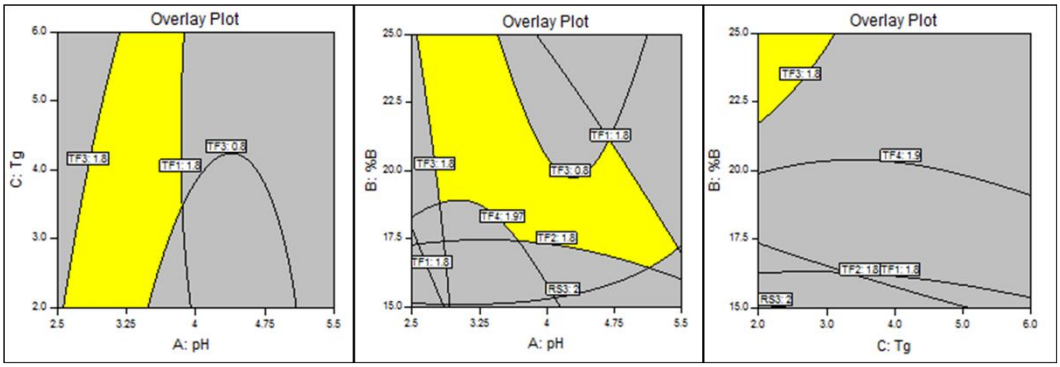


Figure 2.10 Plots for the design space

As shown in Figure 2.10, for plot of Tg versus pH: pH range of 2.5-3.8 was optimum for Tg 2-6. For plot of %B versus pH: %B concentration of 19-25% was found to be optimum for pH range of 2.5-3.5 and %B of 16-21% was optimum for pH 3.5-5.4. For plot of %B versus Tg: %B concentration of 21-25% was optimum for Tg 2-3.

Table 2.7 Gradient Programme

Time(min)	%BI (organic concentration)
0	24
7	24
11	70
18	70
20	50
22	24
24	Stop

Table 2.8 Optimised Parameters

Method parameters	**Optimised value**	
Column	Phenomenex Luna C18 (250mm × 4.6mm i.d × 5µm particle size)	
Mobile phase	Solvent A: 20 mM potassium dihydrogen phosphate buffer (pH = 2.6) Solvent B: ACN Gradient elution as shown in Table 2.7	
Flow rate	1.0 ml /min	
Temperature	Ambient	
Detection wavelength	258 nm	
Retention Time (min)	OFX	4.03
	ORN	9.24
	TBH	15.81
	CBP	19.83
Needle wash	Mobile phase	

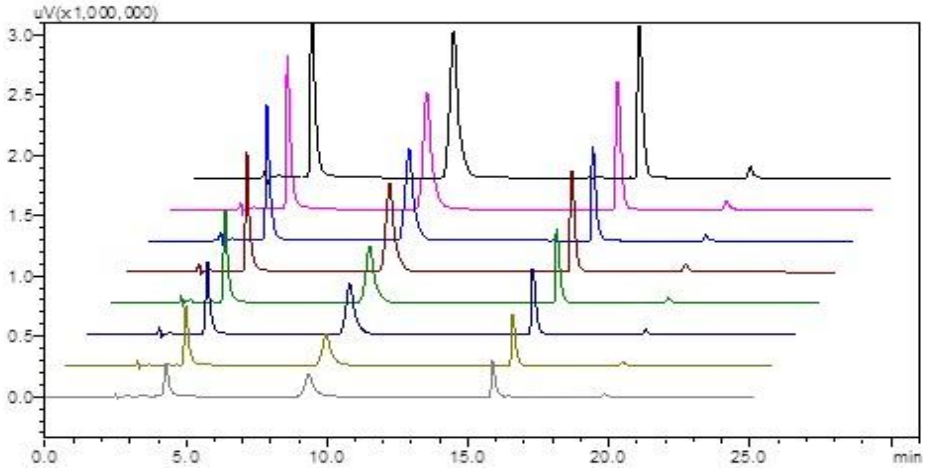
2.6.5. Validation of HPLC method

2.6.5.1. Linearity

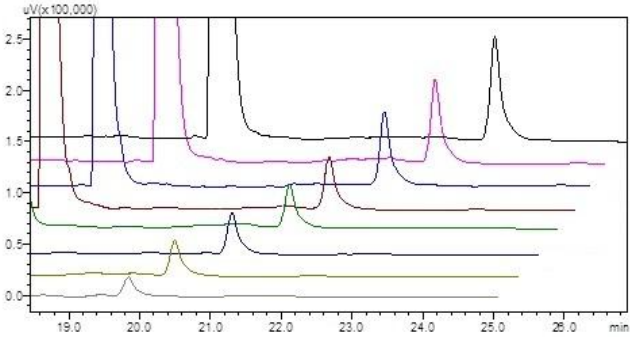
The linearity of the HPLC detector response for determination of OFX, ORN, TBH and CBP was evaluated by the calibration curve for the series of concentrations thus analysed. Characteristic parameters for regression equations of the HPLC method are given in Table 2.9. The overlain chromatograms and calibration curve plots are shown in Figure 2.11 and 2.12 respectively.

Table 2.9 Statistical results of calibration

Parameters	OFX	ORN	TBH	CBP
Calibration range (µg/mL)	150-750	400-2000	200-1000	10-50
Regression equation y=mx+c	y = 21848.67x – 4356.67	y = 13685x – 1214	y = 15408.7x – 2857.3	y = 21466x + 8948
Correlation coefficient(r^2)	0.9989	0.9990	0.9994	0.9991



[A] {OFX 1ST peak, ORN 2ND peak, TBH 3rd peak and CBP 4th peak}



[B] Zoom view of fourth peak of CBP

Figure 2.11 Overlay chromatograms of the standard solutions

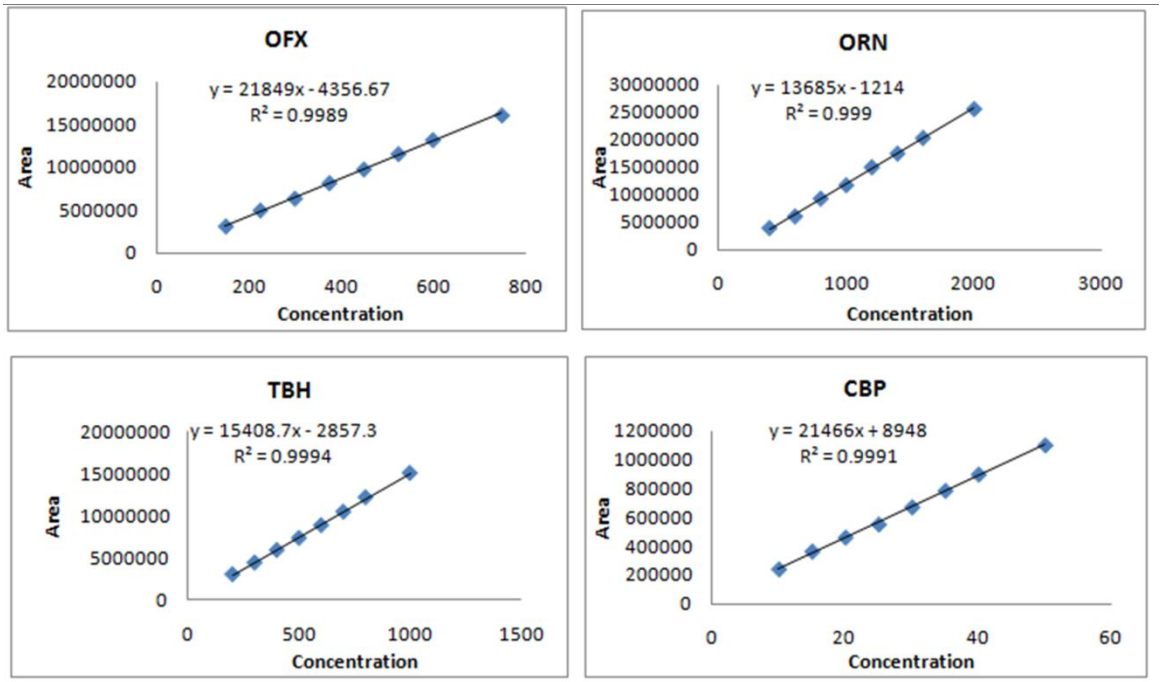


Figure 2.12 Calibration curve plots of OFX, ORN, TBH and CBP [Peak area vs Conc.(µg/mL)]

2.6.5.2. Precision

The precision estimates were evaluated as the average percentage relative standard deviation (% RSD) of intra-day and inter-day measurements for OFX, ORN, TBH and CBP are given in Table 2.10 and 2.11 respectively.

Table 2.10 Intra-day precision studies

DRUG CONC.	PEAK AREA			%RSD	MEAN %RSD
			OFX		
150	2867373	2828436	2903836	1.32	
300	6467274	6392746	6528376	1.05	
450	9791628	9623762	9683746	0.88	
					1.09

		ORN			
400	4069164	4193836	4083948	1.65	
800	9660325	9837534	9773465	0.92	
1200	15019294	15214353	15183847	0.69	
					1.08
		TBH			
200	2783727	2812733	2838722	0.98	
400	6346823	6403635	6392382	0.47	
600	8766361	8693735	8837463	0.82	
					0.76
		CBP			
10	204820	210383	208736	1.37	
20	493655	489321	482938	1.10	
30	678021	663836	673487	1.08	
					1.19

Table 2.11 Inter-day precision studies

DRUG CONC.	**PEAK AREA**			**%RSD**	**MEAN %RSD**
	***DAY1**	***DAY2**	***DAY3**		
		OFX			
150	2867373	2942097	2924745	1.34	
300	6467274	6393570	6333287	1.049	
450	9791628	9967089	9882658	0.89	
					1.09
		ORN			
400	4069164	4111110	4212348	1.78	
800	9660325	9563566	9448371	1.11	
1200	15019294	15151529	14839348	1.04	
					1.32

TBH					
200	2783727	2827019	2873874	1.59	
400	6346823	6299193	6413450	0.90	
600	8766361	8910436	8869837	0.84	
					1.11
CBP					
10	204820	205661	211321	1.71	
20	493655	487220	482594	1.14	
30	678021	682499	693347	1.15	
					1.33

2.6.5.3. Accuracy

The percentage recoveries of the accuracy study are shown in Table 2.12 which suggested good accuracy of the proposed method.

Table 2.12 Recovery results from Cream

Percentage ± standard addition				
OFX				
% Addition	0	80	100	120
Theoretical Content (µg/mL)	150	270	300	330
Amount Found (µg/mL)	148.69	264.75	296.54	329.15
Recovery(%) ± S.D.	99.13 ± 0.84	98.0± 0.5	98.84±0.80	99.74±0.64
ORN				
% Addition	0	80	100	120
Theoretical Content (µg/mL)	400	720	800	880
Amount Found (µg/mL)	398.19	710.84	796.48	878.62
Recovery(%) ± S.D.	99.54 ± 0.69	98.72± 0.75	99.56±0.46	99.84±0.45

TBH				
% Addition	0	80	100	120
Theoretical Content (µg/mL)	200	360	400	440
Amount Found (µg/mL)	198.01	357.57	394.27	440.58
Recovery(%) ± S.D.	99.01 ± 0.95	99.32± 0.25	98.56±1.11	100.13±1.16
CBP				
% Addition	0	80	100	120
Theoretical Content (µg//mL)	10	18	20	22
Amount Found (µg/mL)	9.96	18.15	19.65	21.72
Recovery(%) ± S.D.	99.66± 0.96	100.82±1.25	98.22±1.30	98.72±1.30

2.6.5.4. Detection and quantitation limits

The limit of detection (LOD) and limit of quantitation (LOQ) and values thus found are given in Table 2.13.

Table 2.13 Results of LOD and LOQ

Parameters	OFX	ORN	TBH	CBP
LOD (µg/mL)	0.05	0.2	0.08	0.12
LOQ (µg/mL)	0.14	0.05	0.24	0.35

2.6.5.5. Robustness

The results of robustness are shown in Table 2.14.

Table 2.14 Robustness results

PARAMETERS		OFX	ORN	TBH	CBP
pH	2.4	3.97	9.15	15.78	19.77
	2.6	4.03	9.24	15.81	19.83
	2.8	4.05	9.34	16.09	20.03
	Mean	4.02	9.24	15.89	19.88
	SD	0.042	0.095	0.171	0.136

FR	0.8	5.12	9.97	16.25	20.54
	1	4.03	9.24	15.81	19.83
	1.2	3.42	8.43	15.02	19.01
	Mean	4.19	9.21	15.69	19.79
	SD	0.861	0.770	0.624	0.766
%BI	23	4.49	9.52	16.01	20.14
	24	4.03	9.24	15.81	19.83
	25	3.82	8.98	15.45	19.36
	Mean	4.11	9.25	15.76	19.78
	SD	0.343	0.270	0.284	0.393
Gradient Time (Tg)	3	3.84	8.95	15.736	19.51
	4	4.03	9.24	15.81	19.83
	5	4.30	9.47	16.45	20.20
	Mean	4.06	9.22	16.0	19.84
	SD	0.23	0.26	0.39	0.34

2.6.5.6. Stability

The stability of standard solutions, assessed for 24 h at room temperature and for 48 h in refrigerator (20 ± 2 ºC) showed no additional peak in the chromatogram and no significant reduction in peak intensity was observed which indicated the stability of the standard solutions under study.

2.6.5.7. Specificity

In the commercial formulation there were two labelled excipients *i.e.* propyl paraben (PRP) and methyl paraben (MEP) which gave resolved peaks without interfering the main drugs. This demonstrates the specificity of the method which can be confirmed by comparing the chromatograms of standard solution (Figure 2.8) and sample solution (Figure 2.13). The confirmation of the excipient peaks was done by spiking the excipients (PRP, MEP) and analysing them with the active ingredients. The chromatogramof the sample solution spiked with the excipients has been shown in Figure 2.14.

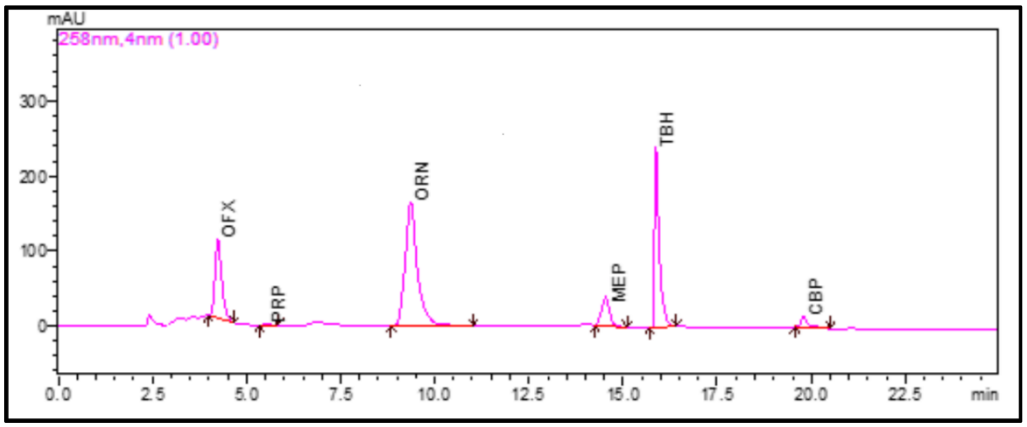


Figure 2.13 Chromatogram of sample solution mixture containing 150 ppm of OFX, 400 ppm of ORN, 10 ppm of CBP and 200 ppm of TBH

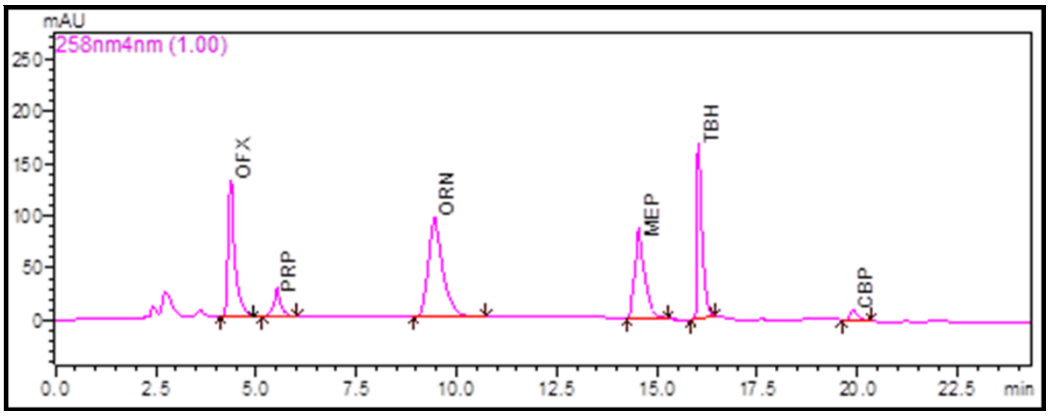


Figure 2.14Chromatogram of sample solution mixture containing OFX, ORN, CBP and TBH spiked with standard solutions of MEP and PRP

2.6.5.8. System suitability

Satisfactory results were obtained for system suitability study of the HPLC method as shown in Table 2.15. The peak purity curves have been shown in Figure 2.15 and the peak purity results are given in Table 2.16.

Table 2.15 System Suitability Parameters for the developed HPLC method

Parameters	OFX	ORN	TBH	CBP
Retention Time	4.03±1.90	9.24±1.33	15.81±0.49	19.83±0.63
Tailing factor	1.37±1.82	1.39±1.18	1.78±1.44	1.69±1.42
Resolution	--	12.98±1.51	15.96±1.08	13.34±1.87
Theoretical Plates	4342.28±1.71	4792.34±1.95	49971.43±1.84	79732.02±1.75

Mean±standard deviation for n=6 replicates

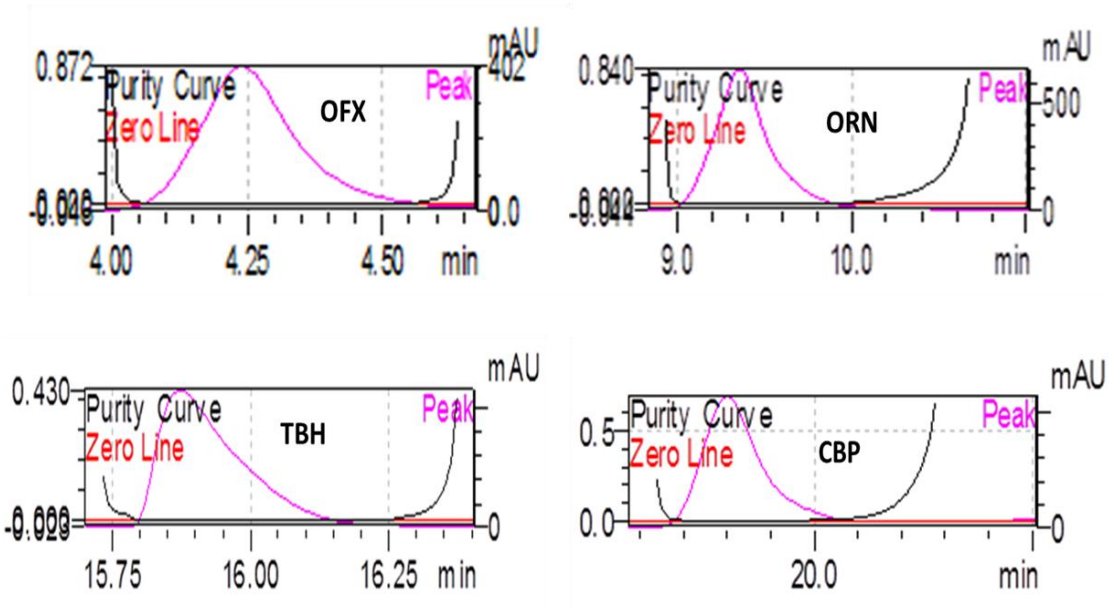


Figure 2.15The peak purity curves of all four drugs

Table 2.16 Peak Purity results

Drug Name	Peak Purity Index	Threshold
OFX	1.0000	0.9999
ORN	1.0000	0.9999
TBH	0.9999	0.9999
CBP	1.0000	0.9997

2.6.6. Summary of validation parameters

The results of the HPLC method validation have been summarised in Table 2.17.

Table 2.17 Summary of validation parameters

Parameters	OFX	ORN	TBH	CBP
Calibration range (µg/mL)	150-750	400-2000	200-1000	10-50
LOD (µg/mL)	0.05	0.2	0.08	0.12
LOQ (µg/mL)	0.14	0.05	0.24	0.35

Regression equation	y = 21848.67x – 4356.67	y = 13685x – 1214	y = 15408.7x – 2857.3	y = 21466x + 8948
Correlation coefficient(r^2)	0.9989	0.9990	0.9994	0.9991
Accuracy (% Recovery±SD)*				
80%	98.0± 0.5	98.72± 0.75	99.32± 0.25	100.82±1.25
100%	98.84±0.80	99.56±0.46	98.56±1.11	98.22±1.30
120%	99.74±0.64	99.84±0.45	100.13±1.16	98.72±1.30
Precision(%RSD)*				
Intraday	1.08	1.089	0.76	1.18
Interday	1.09	1.32	1.11	1.33

* Average of three replicates

2.6.7. Analysis of marketed formulation

The RP-HPLC method was successfully applied to the determination of OFX, ORN, TBH and CBP in cream formulation without the interference of excipients therein. The results of the assay are shown in Table 2.18.

Table 2.18 Results for assay of cream formulation

	OFX	**ORN**	**TBH**	**CBP**
Label claim (%w/w)	0.75	2	1	0.05
% Assay±SD[#]	99.74± 0.39	98.72± 0.71	98.19 ±0.23	99.05 ±0.76

[#]Determination for n=6 replicates

2.6.8. Permeability study

Permeability study was carried out using Franzch diffusion cell, in various media such as physiological buffer solution (PBS) and normal saline solution. The release for CBP was very less in these media. The reported literature (40, 41) suggested addition of ethanol to increase the release and hence ethanol was added to the PBS media upto

30% to enhance release of CBP. The permeability study was continued for upto 24 hrs and the cumulative percentage release was calculated. The results are shown in Table 2.19. Figure 2.16 shows the plot of cumulative percentage release of the drugs versus time profile.

Table 2.19 Results for Permeability study

Time (HRS)	Cumulative Percentage Release (CPR)			
	OFX	ORN	TBH	CBP
1	7.5 ± 0.84	6.2 ± 0.48	5.1 ± 0.35	0.0 ± 0
2	16.0 ± 1.53	14.7 ± 0.71	10.2 ± 1.05	3.5 ± 0.64
3	25.0 ± 1.47	21.2 ± 1.53	16.1 ± 1.61	8.8 ± 0.52
4	35.2 ± 1.10	29.8 ± 1.35	23.6 ±1.90	16.1 ± 0.86
5	45.9 ± 1.5	39.4 ± 1.81	32.3 ± 1.35	25.4 ± 0.95
6	57.4 ± 1.24	50.7 ± 1.98	42.3 ± 1.73	35.4 ± 1.77
7	69.2 ± 0.98	62.6 ± 1.26	54.1 ± 1.91	46.7 ± 1.62
22	82.0 ± 1.43	75.1 ± 1.9	66.4 ± 0.84	61.4 ± 1.75
24	94.9 ± 1.26	88.2 ± 1.5	77.4 ± 1.77	82.3 ± 1.98

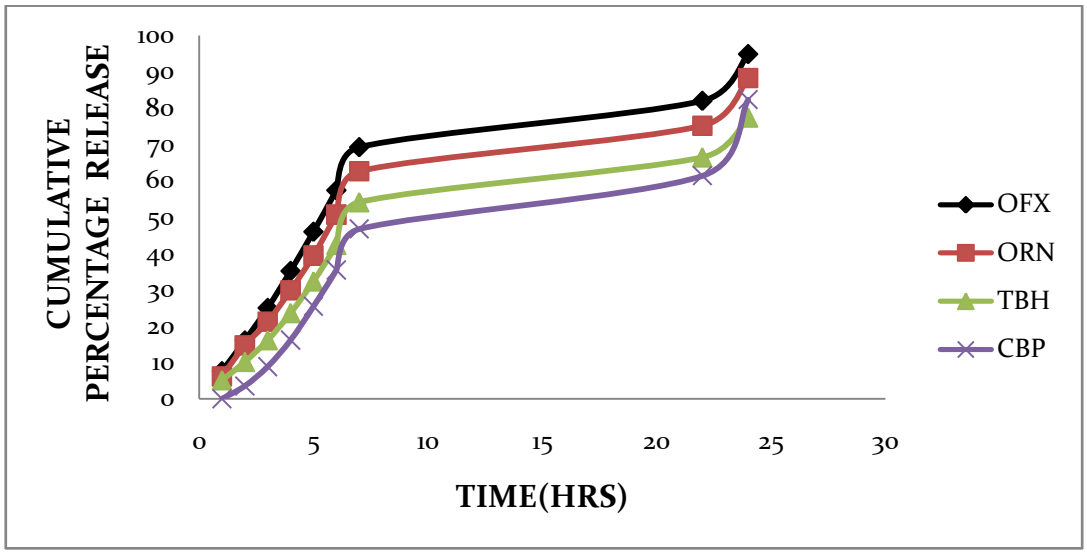


Figure 2.16 Plot of percentage permeability with respect to time in hours (HRS)

2.7. CONCLUSION

The QbD approach was successfully applied for the optimisation and development of RP-HPLC method for the simultaneous estimation of ofloxacin, ornidazole, terbinafine maleate and clobetasol propionate, in cream formulation, wherein full factorial design was used for finding out the most suitable conditions giving best separation of the four components within shortest possible time period and appropriate SST parameters. The optimised RP-HPLC method has been applied for estimation of the four components in cream formulation and also to estimate their *invitro* permeability through rat skin.

2.8. REFERENCES

1. http://www.drugbank.ca/drugs/DB01165. Accessed on: 21 July 2015
2. http://www.genericpedia.com/generic/ornidazole. Accessed on: 21 July 2015
3. http://www.rxlist.com/lamisil-drug/clinical-pharmacology.html. Accessed on: 21 July 2015.
4. http://pubchem.ncbi.nlm.nih.gov/compound/clobetasol_propionate#section=Top. Accessed on: 21 July 2015.
5. http://www.chemspider.com/Chemical-Structure.26102.html. Accessed on: July 2015
6. http://www.drugbank.ca/drugs/DB00857. Accessed on: 23 July 2015
7. http://www.drugbank.ca/drugs/DB01013. Accessed on: 23 July 2015
8. www.chemACX.com (Cambridge database). Accessed on: 23 July 2015
9. Kassem H, Almardini MA. High Performance Liquid Chromatography method for the determination of terbinafine hydrochloride in semi solids dosage form. Int J Pharm Sci Rev Res. 2013; 21(2): 58-61.
10. Patel K. A validated RP-HPLC method for determination of terbinafine hydrochloride in pharmaceutical solid dosage form. Int J Pharm Technol. 2012; 4(3): 4663-9.
11. Cardoso SG, Elfrides ES, Schapoval. High-performance liquid chromatographic assay of terbinafine hydrochloride in tablets and creams. J Pharm Biomed Anal 1999; 19: 809-12.

12. Ramesh RR., Bujji BN. Simultaneous analysis of RPHPLC method development and validation of terbinafine and bezafibrate drugs in pharmaceutical dosage form. Pharmacophore. 2011; 2 (4): 232-8.
13. Tagliari MP, Kuminek G, Sílvia HM, Borgmann, Charise BD, Cardoso SG. Terbinafine: optimization of a LC method for quantitative analysis in pharmaceutical formulations and its application for a tablet dissolution test. Quim Nova.2010; 33(8): 1790-3.
14. Fontana MC, Bastos MO, Beck RCR. Development and validation of a fast RP-HPLC method for the determination of clobetasol propionate in topical nanocapsule suspensions. J Chromatogr Sci. 2010; 48: 637-40.
15. Gagliardia L, De DO, Manna F, Tonelli D. HPLC determination of clobetasol propionate in cosmetic products. J Liq Chromatogr R T. 2000; 23(3): 355-62.
16. Siva Kumar R, Kumar NP, Saravanakumar S, Kandasamy CS, Venkatnarayanan R. Simultaneous RP-HPLC Estimation of Nitazoxanide and Ofloxacin in tablet dosage forms. Asian J Res Chem. 2009; 2(1): 43-5.
17. Puranik M, Bhawsar DV, Rathi P, Yeole PG. Simultaneous determination of Ofloxacin and Ornidazole in solid dosage form by RP-HPLC and HPTLC techniques. Indian J Pharm Sci. 2010; 72(4): 513-17.
18. Narayana Reddy K VVS, Suresh Kumar R, Hemnath E, Venkata ST, Karthik Yamjala K, Malayand R. Development and validation of RP-UFLC method for the estimation of Terbinafine HCl in pharmaceutical nanoemulsion gel. Int J Pharm PharmSci. 2014; 6(5): 586-90.
19. Shah MN, Patel HU, Patel CN. A validated Stability-indicating HPLC method for determination of ofloxacin and cefpodoxime proxetil in tablet dosage form. Inventi Rapid: Pharm Analysis & Quality Assurance 2012; Article ID-Inventi:ppaqa/380/12.
20. Burana-Osot J, Saowakul K, Charoensilpchai C, Surapeepong N, Pongsiriwan W, Kumsum A, Zigic ZG, Zecevic M. Stability-indicating HPLC method for determination of ofloxacin in bulk drug and tablets using trifluoroacetate as counter anions. J Liq Chromatogr R T. 2012; 35: 1909-19.
21. Huang J, Cao G, Hu X, Sun C, Zhang J. Chiral Separation of rac-Ornidazole and detection of the impurity of (R)-Ornidazole in (S)-Ornidazole injection and raw material. Chirality. 2006; 18: 587-91.

22. Penmatsa VK, Basavaiah K. Simple, sensitive and stability indicating high performance liquid chromatographic assay of terbinafine hydrochloride in dosage forms. Am J PharmTech Res. 2014; 4(2): 899-916.
23. Ahmad S, Jain GK, Md Faiyazuddin, Iqbal Z, Talegaonkar S, Sultana Y, Ahmad FJ. Stability-Indicating High-Performance Thin- Layer Chromatographic method for analysis of Terbinafine in pharmaceutical formulations. ActaChromatogr.2009; 21(4): 631-9.
24. Goswami PD. Stability-indicating RP-HPLC method for analysis of terbinafine hydrochloride in bulk and in tablet dosage form. Int J PharmPharm Sci. 2013; 5(3): 536-40.
25. Md. Ali S, Md. Alam S, Alam N, Safhi MM. Accelerated stability testing of clobetasol propionate loaded nanoemulsion as per ICH guideline. Sci Pharm. 2013; 81: 1089-1100.
26. Fauzee AF, Walker RB. Forced degradation studies of clobetasol 17-propionate in methanol, propylene glycol, as bulk drug and cream formulations by RP-HPLC. J Sep Sci. 2013; 36(5): 849-56.
27. Gagliardi L, Orsi D, Giudice MR, Gatta F, Porrà R, Chimenti P, Tonelli D. Development of a tandem thin-layer chromatography-high-performance liquid chromatography method for the identification and determination of corticosteroids in cosmetic products. Anal Chim Acta. 2002; 457: 187-98.
28. Patel KK, Karkhanis VV. A validated HPTLC method for determination of terbinafine hydrochloride in pharmaceutical solid dosage form. Int J Pharm SciRes. 2012; 3(11): 4492-5.
29. Suma BV, Kannan K, Madhavan V, Nayar CR. HPTLC method for determination of Terbinafine in the bulk drug and tablet dosage form. Int. J. ChemTech Res. 2011; 3(2): 742-8.
30. Jain PS, Chaudhari AJ, Patel SA, Patel ZN, Patel DT. Development and validation of the UV-spectrophotometric method for determination of terbinafine hydrochloride in bulk and in formulation. Pharm Methods. 2011; 2(3): 198-202.
31. Goswami PD. Validated spectrophotometric method for the estimation of terbinafine hydrochloride in bulk and in tablet dosage form using inorganic solvent. Der Pharm Lett.2013; 5(3):386-90.
32. Chennaiah M, Veeraiah T, Vinod Kumar T, Venkateshwarlu G. Extractive spectrophotometric methods for determination of terbinafine hydrochloride in

pharmaceutical formulations using some acidic triphenylmethane dyes. Indian J Chem Technol. 2012; 19: 218-21.

33. Cardoso SG, Schapoval EE.UV spectrophotometry and nonaqueous determination of Terbinafine Hydrochloride in dosage forms. J AOAC Int.1999; 82(4): 830-3.
34. Abou-elkheir A, Saleh H, El-henawee MM. Spectrophotometric determination of TerbinafineHCl, Telmisartan and Ramipril through redox reactions using ceric sulphate and ceric sulphate-chromatrope2R. Int J Pharm Chem Biol Sci. 2014; 4(4): 931-43.
35. Mikus P, Valaskova I, Havranek E. Determination of terbinafine in pharmaceuticals and dialyzates by capillary electrophoresis. Talanta. 2005; 65: 1031-7.
36. Dewani AP, Bakal RL, Kokate PG, Chandewar AV, Patra S.Development of a Single Ion Pair HPLC method for analysis of Terbinafine, Ofloxacin, Ornidazole, Clobetasol and two preservatives in a Cream formulation: Application to in vitro drug release in topical simulated media phosphate buffer through rat skin. J AOAC Int. 2015; 98(4): 913.
37. Amipara M, Patel S, Pathan S, Patel J, Kakadiya J, Shah N. Development and validation of RPHPLC method for the estimation of terbinafine hydrochloride, clobetasol propionate, ornidazole and ofloxacin in their combined semisolid pharmaceutical dosage. Inveti Rapid Pharm Anal Qual Assur. 2015; Article ID: Inventi:ppaqa/16221/15.
38. Bommadevara P, Rahaman SA. Validated stability indicating RPHPLC method for the simultaneous determination of ofloxacin, ornidazole, clobetasol propionate and terbinafine hydrochloride, methyl paraben, propyl paraben in bulk and pharmaceutical dosage form. Int J Pharm Anal Res. 2014; 3(4): 301-18.
39. ICH guidelines, validation of analytical procedure: Methodology Q2B; I.C.H. Harmonized Tripartite Guidelines. Geneva; 1996.
40. Tsai J. Content and Transdermal Delivery of Clobetasol 17-Propionate from Commercial Creams and Ointments. J Food Drug Anal. 2012; 10(1): 7-12.
41. Badilli U, Sen T and Tarimci N. Microparticulate based topical delivery system of Clobetasol Propionate. AAPS Pharm SciTech. 2011; 12(3): 949-57.

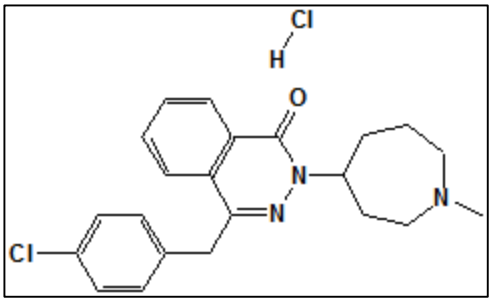
DEVELOPMENT OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF AZELASTINE HYDROCHLORIDE AND BUDESONIDE IN BULK DRUG AND LABORATORY MIXTURE: QbD BASED OPTIMISATION

3.1. SELECTION OF FORMULATION

The marketed formulation i.e. the nasal spray of including the combination of Azelastine hydrochloride (AZ) and Budesonide (BD) named as CDX-313 by Cydex Pharma, is under Phase II clinical trials (1). A combination of Captisol-Enabled BD and AZ (CDX-313) has been proved to provide fast and long-lasting relief in the symptoms of allergic rhinitis (2-4). Captisol is a cyclodextrin derivative compound and literature reveals that aqueous nasal suspension with cyclodextrin as well as its derivative enhances pharmacological efficacy of drug. It improves the drug retention in nasal mucosa, and increases its duration of action so that the frequency of administration decreases which can improve patient compliance (5). However nasal sprays of individual drugs of AZ and BD are available in market, namely Rhinocort Aqua® for BD and Astelin® for AZ. Thus due to the promising actions of both drugs and their benefits in combination it can be expected to clear the clinical trials and thus this combination was selected for study.

3.2. DRUG PROFILE

(1) Azelastine hydrochloride (AZ) (6, 8)



a. **Category** **:** Antihistaminic
b. **Molecular formula :** $C_{22}H_{24}ClN_3O.HCl$
c. **Molecular Weight :** 418.37
d. **Nomenclature:** (±)4-(4-chlorobenzyl)-2-(1-methylazepan-4-yl)-phthalazin-1-(2H)-one hydrochloride

e. Physicochemical Properties:

i. Description: white powder

ii. Solubility: Soluble in MeOH; sparingly soluble in water and ACN

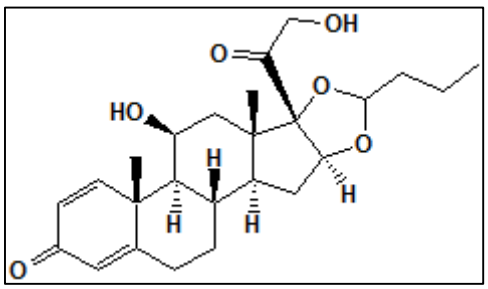
iii. log P: 4.0

iv. pKa: 8.88

v. Melting Point: 225-227 º C

f. Official Status: Official in IP, USP, EP and BP.

(2) Budesonide (BD) (7, 8)



a. Category : Anti-inflammatory corticosteroid

b. Molecular formula : $C_{25}H_{34}O_6$

c. Molecular Weight : 430.5

a. Nomenclature:(11beta,16alpha)-16,17-(butylidenebis(oxy))-11,21dihydroxypreg na-1,4-diene-3,20-dione

b. Physicochemical Properties:

i. Description: off-white powder

ii. Solubility: Soluble in MeOH; slightly soluble in ACN; insoluble in water

iii. log P: 2.73

iv. pKa: 13.74, -2.9

v. Melting Point: 224-226 ºC

c. Stereochemistry :BD occurs as a mixture of two epimers (22R and 22S)

d. Official Status: Official in IP, USP, EP and BP.

3.3. MARKETED FORMULATION

The studies carried out for this formulation provide a reference for the doses of AZ and BD as 137µg/mL and 32 µg/mL resp. (2). As the nasal spray formulation of AZ and BD is under clinical trials, a laboratory mixture with reference to literature (9) was prepared and used for formulation study. The formula is given in Table 3.1.

Table 3.1 Formula for the laboratory mixture

Ingredient	Quantity (mg/100mL)
AZ	274
BD	64
Captisol	5000
Sodium citrate dibasic trihydrate	44
Citric acid	32
Sodium chloride	480
Sodium EDTA	50
Polysorbate 80	50
Water	QS(upto 100 mL)

3.4. LITERATURE REVIEW

Various analytical methods have been reported in the literature for the quantitation of AZ and BD either individually or in various combinations with other drugs using UV spectrophotometric method (10-12), spectrophotometric determination and thermodynamic studies of Azelastine-HCl complexes (13), RP-HPLC methods (14-19), LC-MS method for BD (20) and thermoanalytical study and purity determination of AZ (21). There is no analytical method in the literature for the simultaneous estimation of AZ and BD. The present study deals with the QbD approach for RP-HPLC method development for the simultaneous estimation of AZ and BD. The method thus optimised has been applied for the estimation of AZ and BD in bulk drug and laboratory mixture.

3.5. PLACKETT-BURMAN: QUALITY BY DESIGN APPROACH FOR SCREENING STAGE

3.5.1. EXPERIMENTAL

3.5.1.1. Instrumentation

Chromatography was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AD pump (binary) and Shimadzu PDA-M20A Diode Array Detector. Samples were injected through a Rheodyne 7725 injector valve with fixed loop at 20 µL. Data acquisition and integration were performed using LC Solution software (Shimadzu Corporation, Kyoto, Japan). Separation and quantitation were made on a PhenomenexC18 column (5µm × 250mm × 4.6mm i.d.). The experimental design was performed on Minitab® 16.2.1software.

3.5.1.2. Materials and reagents

The API of AZ was procured from Sigma Aldrich and BD was gifted by Avik Pharmaceuticals Ltd., Vapi Gujarat. HPLC grade (Spectrochem) MeOH and ACN were used for HPLC. Double distilled water, AR grade potassium dihydrogen phosphate (LobaChem), AR grade phosphoric acid (LobaChem), HPLC grade triethylamine (Spectrochem), AR grade ammoniun formate (LobaChem) and HPLC grade formic acid (Spectrochem) were used for preparation of buffer.

3.5.1.3. The Design of Experiments

The critical (or major) responses selected for the design which affect the process performance were studied and an optimum range in which the chromatographic parameters had maximum effect were analysed and studied before beginning of the design. A Plackett-Burman screening design was applied to investigate the significance of the seven selected factors. The factors have been listed in Table 3.2. Seven responses were studied, namely resolution between AZ and BD (RS), retention time of AZ (RTAZ) and BD (RTBD), tailing factor of AZ (TFAZ) and BD (TFBD) and theoretical plates of AZ (TPAZ) and BD (TPBD). The summary of Plackett-Burman design has been shown in Table 3.3 and the experimental runs have been shown in Table 3.4.

Table 3.2 Factors screened by Plackett-Burman design

	Variable (Factors)	Acceptable Range
Categorial Factors	Column	C8, C18
	Buffer Type	Ammonium formate buffer (AFB), Phosphate buffer (PB)
Numerical Factors	pH	3-5
	Flow Rate (mL/min)	0.8-1.2
	Temperature (ºC)	25-40
	Buffer Concentration (mM)	10-40
	Organic concentration (%)	40-60

Table 3.3 Summary of Plackett-Burman screening design

Factors	Responses	Replicates	Base blocks	Total blocks	Base runs	Total runs
7	7	1	1	1	12	12

Table 3.4 The experimental runs for the screening design

RUNS	Buffer	Column	Temp	pH	Flow Rate	Organic Conc.	Buff Conc.
1	PB	C18	25	5	1.2	40	40
2	AFB	C8	25	5	1.2	60	10
3	AFB	C18	25	3	0.8	60	40
4	AFB	C8	40	5	1.2	40	40
5	PB	C8	40	3	0.8	40	40
6	PB	C8	40	5	0.8	60	10
7	AFB	C18	40	5	0.8	60	40
8	PB	C18	25	5	0.8	40	10
9	AFB	C18	40	3	1.2	40	10
10	AFB	C8	25	3	0.8	40	10
11	PB	C18	40	3	1.2	60	10
12	PB	C8	25	3	1.2	60	40

3.5.2. RESULT AND DISCUSSION

3.5.2.1. Screening of factors

Development of analytical method depends on a number of factors which directly or indirectly affect the performance of the method. Hence, the first step to QbD is to identify the variables affecting our critical responses and their effects. The "cause and effect" diagram known as the Ishikawa diagram is shown in Figure 3.1. Various factors discussed in the Ishikawa fish bone diagram were studied and their influence on the critical process parameters was studied. Seven important effects like pH, type of buffer, temperature, flow rate, buffer ratio and strength of buffer, etc. were then screened by PBD.

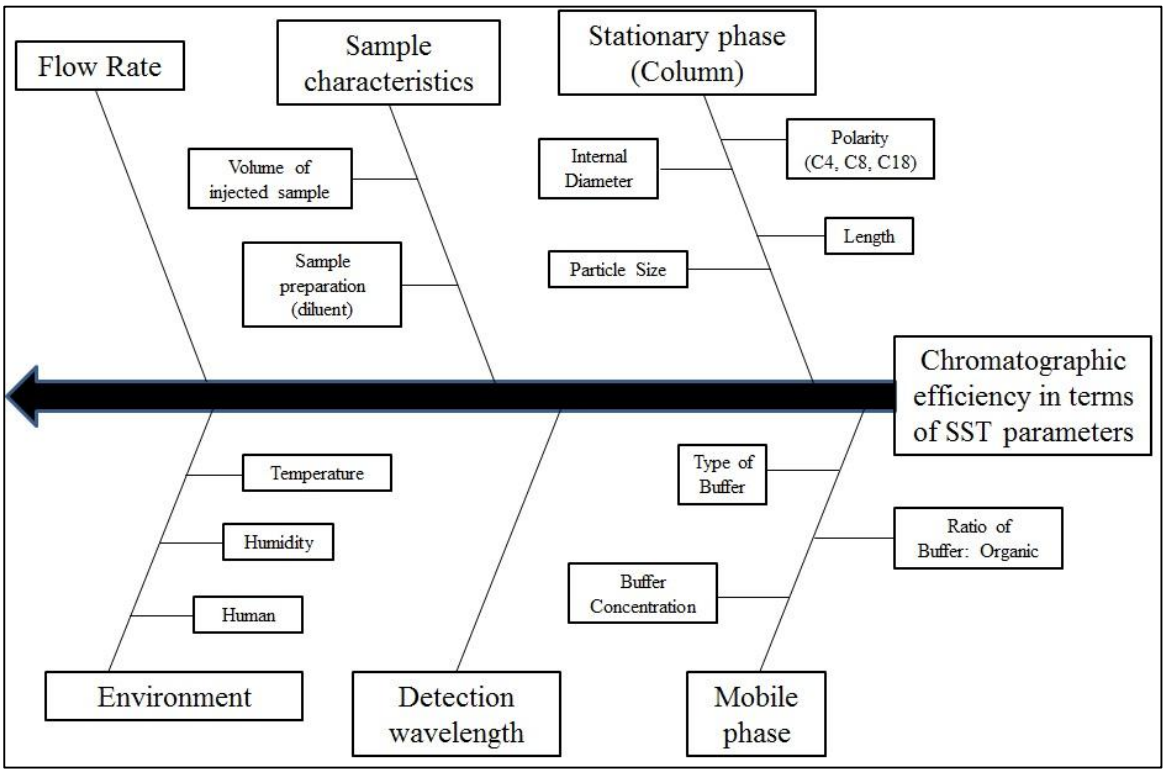


Figure 3.1 Ishikawa Diagram

Based on the overlay of the zero order UV spectra of AZ and BD as shown in Figure 3.2, the detection wavelength was selected as 236 nm.

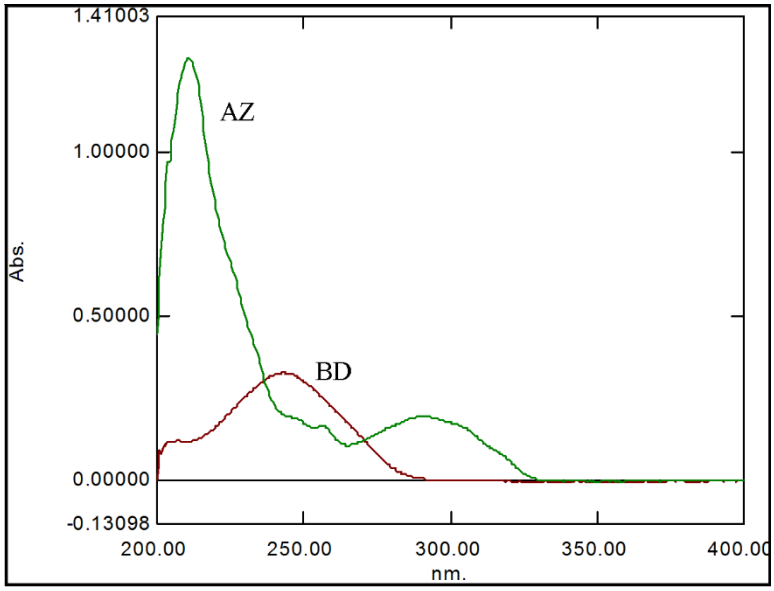


Figure 3.2 The overlay of zero order UV spectra of AZ and BD

The chromatograms of various PBD trials have been shown in Figure 3.3.

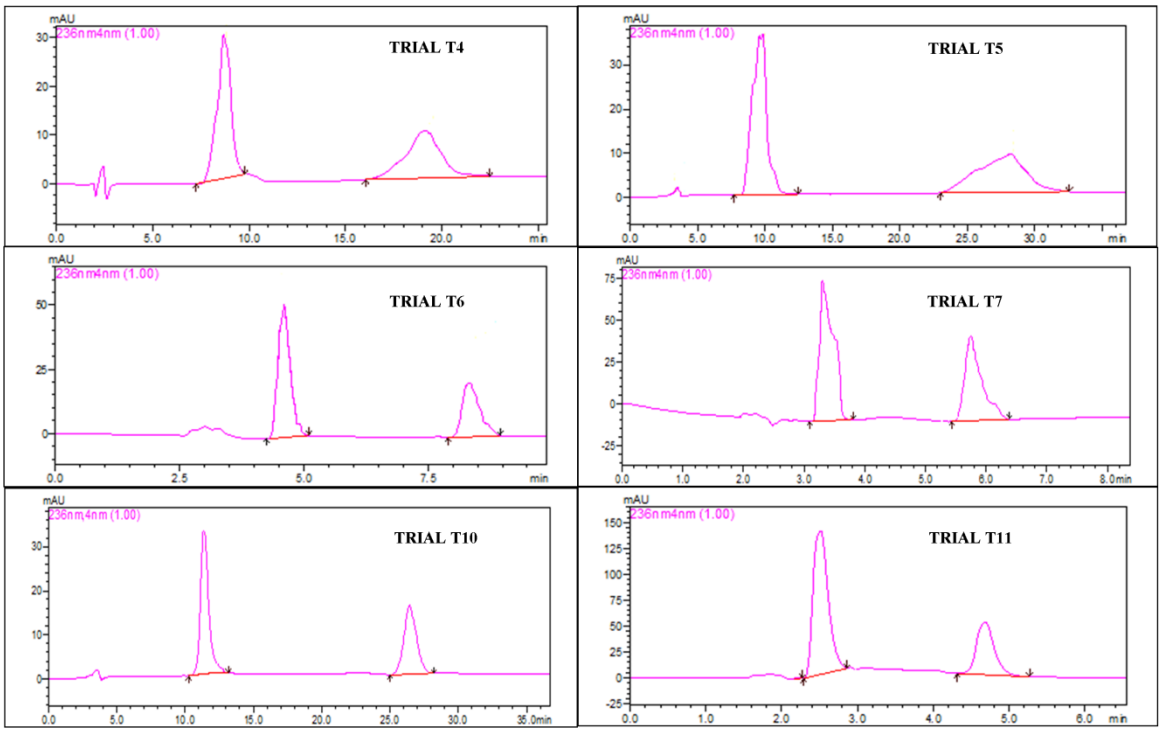
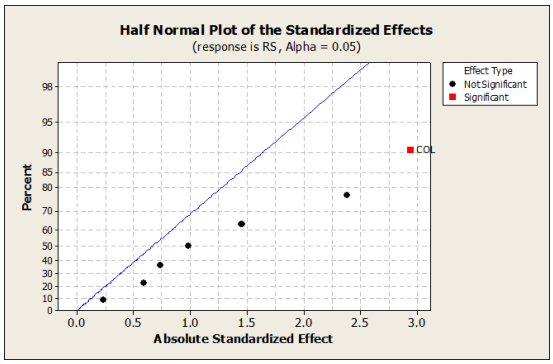


Figure 3.3 Chromatograms of few trials of Plackett-Burman Design (First Peak of AZ and second peak of BD)

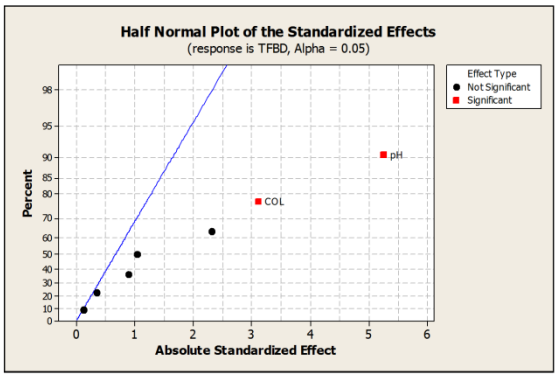
3.5.2.2. The diagrammatic outputs of Plackett-Burman design

(A) Half normal plots

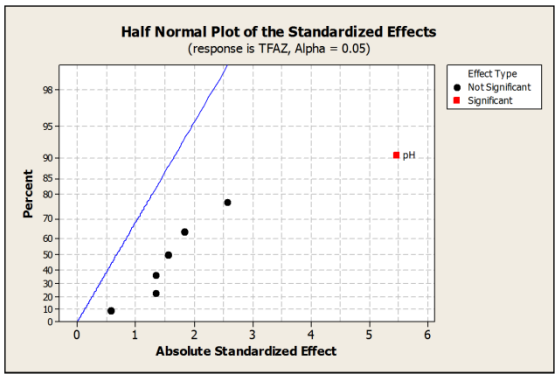
Half normal effects compare the magnitude and the statistical significance of both main and interaction effects. Points that are away from the zero line usually indicate significant effects. Significant effects are labelled and fall away from the line on the right side. The software (Minitab) uses the a-level of 0.05 and labels any effect that is significant. Figure 3.4 shows the half normal plots for all the responses.



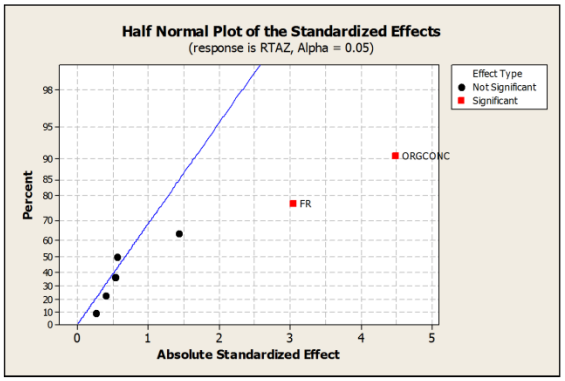
RS

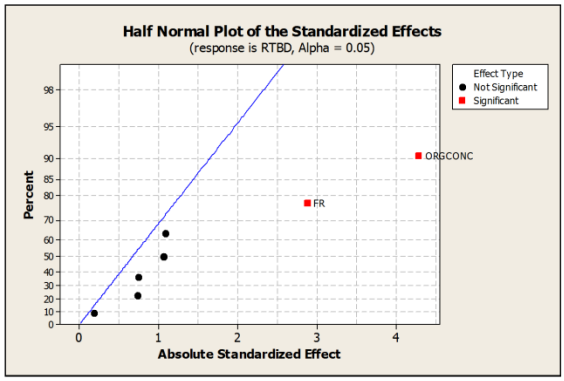


TFBD

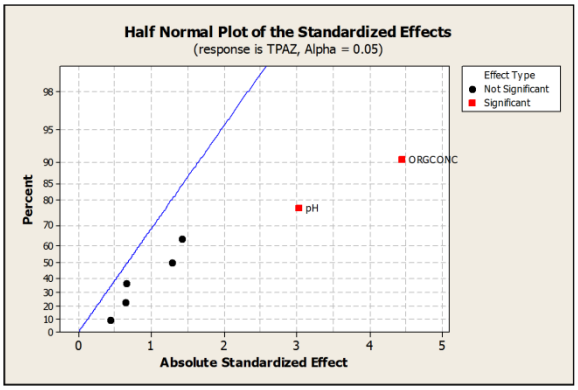


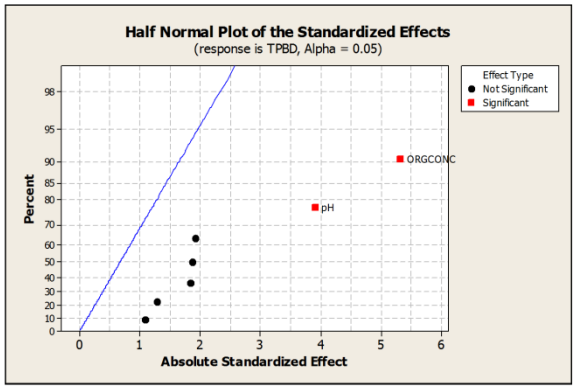
TFAZ





RTAZ **RTBD**





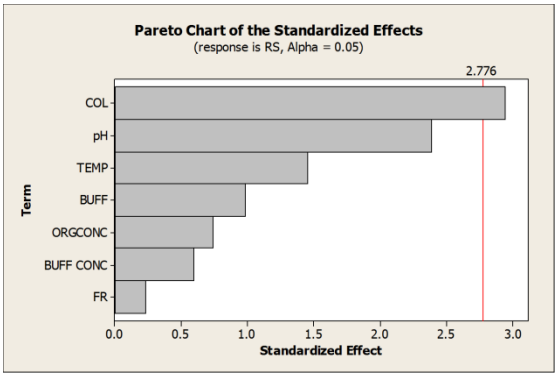
TPAZ **TPBD**

Figure 3.4 Half Normal Plots showing the prominent factors affecting the design optimisation

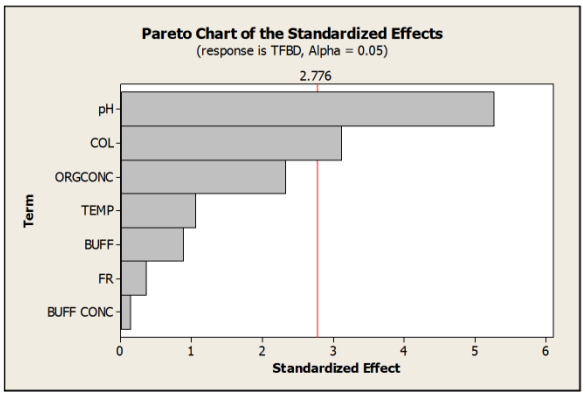
From the Half normal plots it was found that pH, organic concentration, flow rate and type of column were the significant factors affecting the design.

(B) Pareto charts

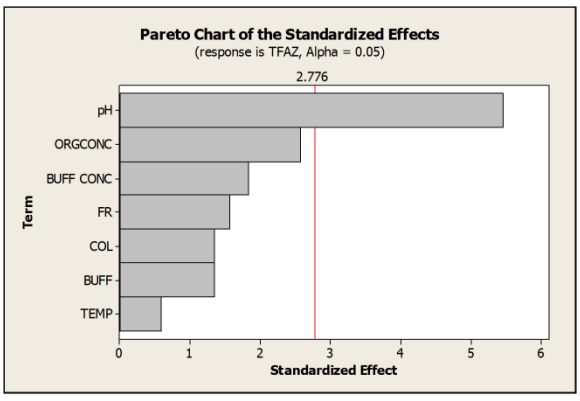
Pareto chart compare the relative magnitude and the statistical significance of both main and interaction effects. The effects are plotted in decreasing order of the absolute value of the effects. The reference line on the chart indicates which effects are significant. The reference line is plotted with an α-level of 0.05. Figure 3.5 shows the pareto charts for all the responses.



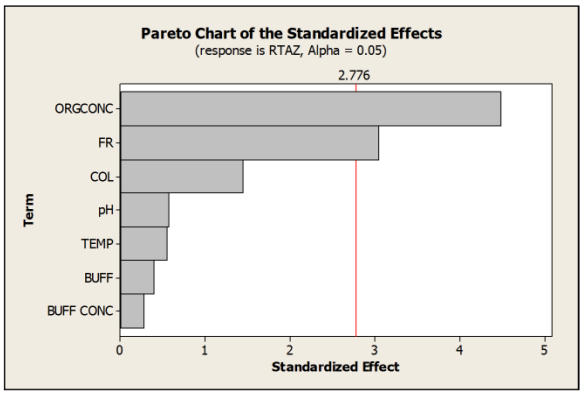
RS



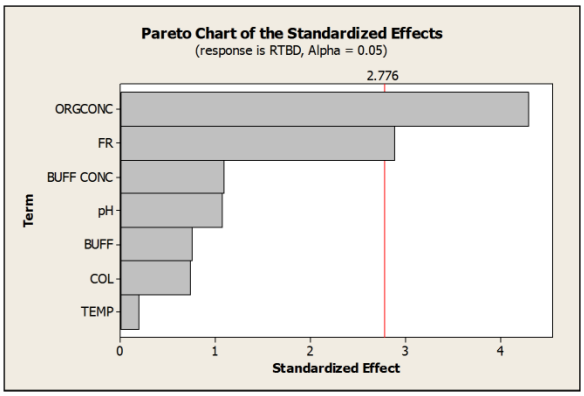
TFBD



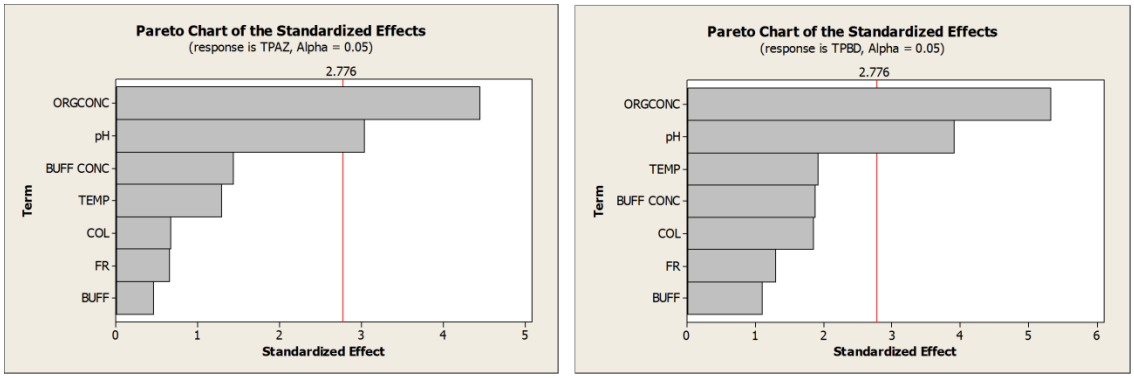
TFAZ



RTAZ



RTBD



TPAZ **TPBD**

Figure 3.5 Pareto Charts showing effects of various factors on various responses

From the pareto charts it was found that pH, organic concentration and flow rate were significant factors affecting various responses.

(C) Residual Plots

The difference between observed response and fitted response value is said to be its residual. Standardized residuals generally have a variance of 1. Standardized residuals with absolute value >2 are considered very large and treated as unusual observations.

The four-in-one residual plot displays four different residual plots together in one graph window. This layout can be useful for comparing the plots to determine whether the model meets the assumptions of the analysis. The residual plots in the graph include:

- Histogram which indicates whether the distribution of data [whether skewed or outliers exist in the data]. The histogram of the residuals should be bell-shaped.
- Normal probability plot that indicates whether the data are normally distributed, other variables are influencing the response or outliers exist in the data. The normal probability plot of the residuals should roughly follow a straight line.
- Residuals versus fitted values which indicate the type of variance [constant or non-linear or outliers exist in the data]. The residuals should be scattered randomly about zero. A point far away from zero is an outlier.
- Residuals versus order of the data which indicates if any systematic effects are there in the data due to time or data collection order. This graph plots the residuals in the order of the corresponding observations. The plot is useful when

the order of the observations may influence the results. The residuals in the plot should fluctuate in a random pattern around the centre line.

Figure 3.6-3.12 shows the residual plots for all the responses.

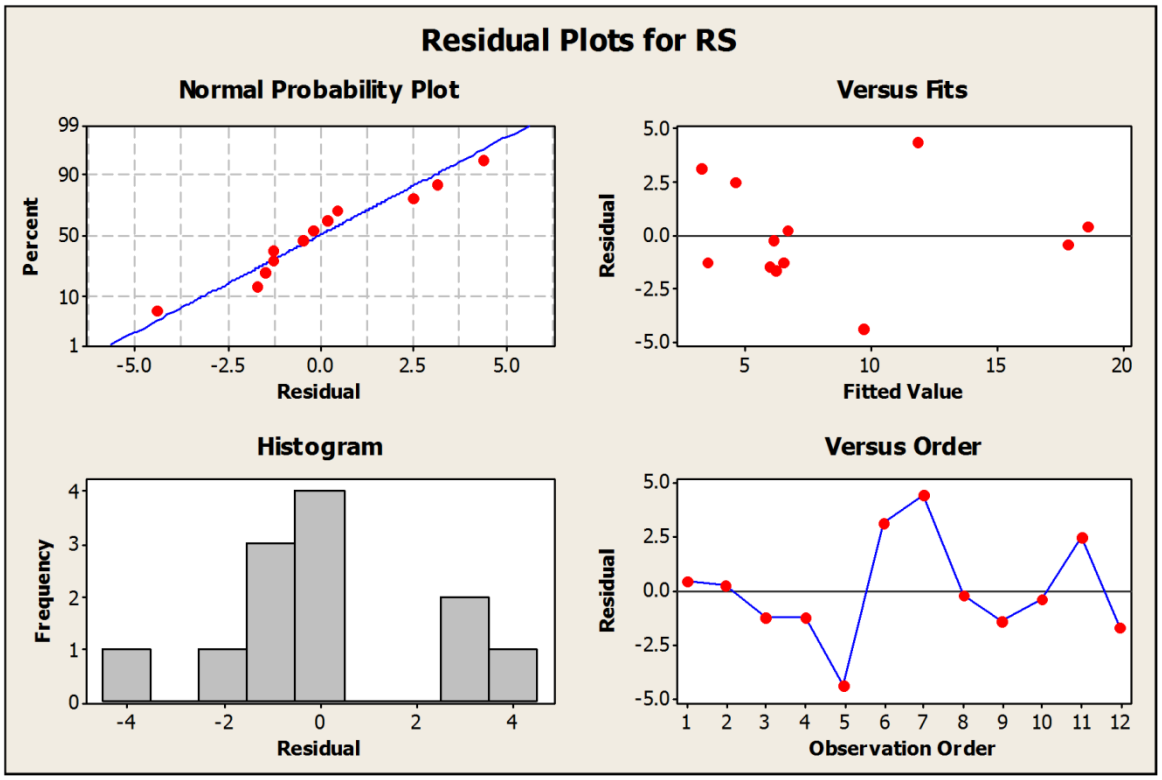


Figure 3.6 Residual Plots for RS

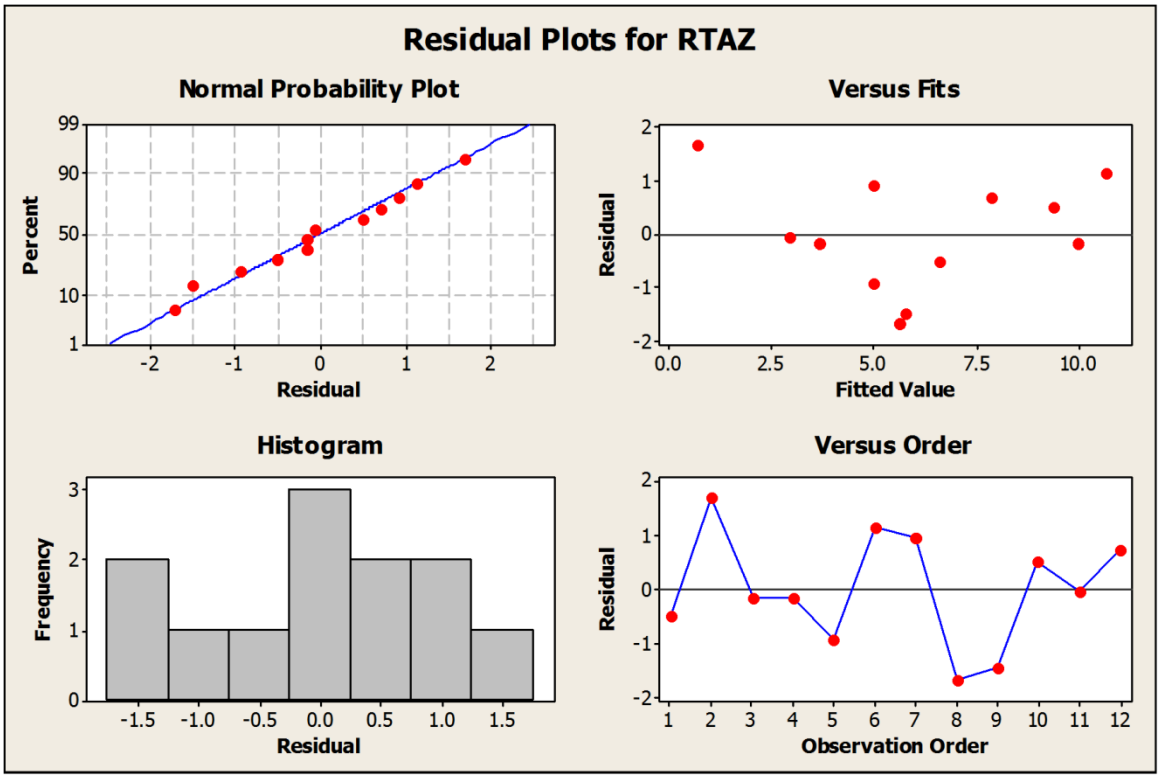


Figure 3.7 Residual Plots for RTAZ

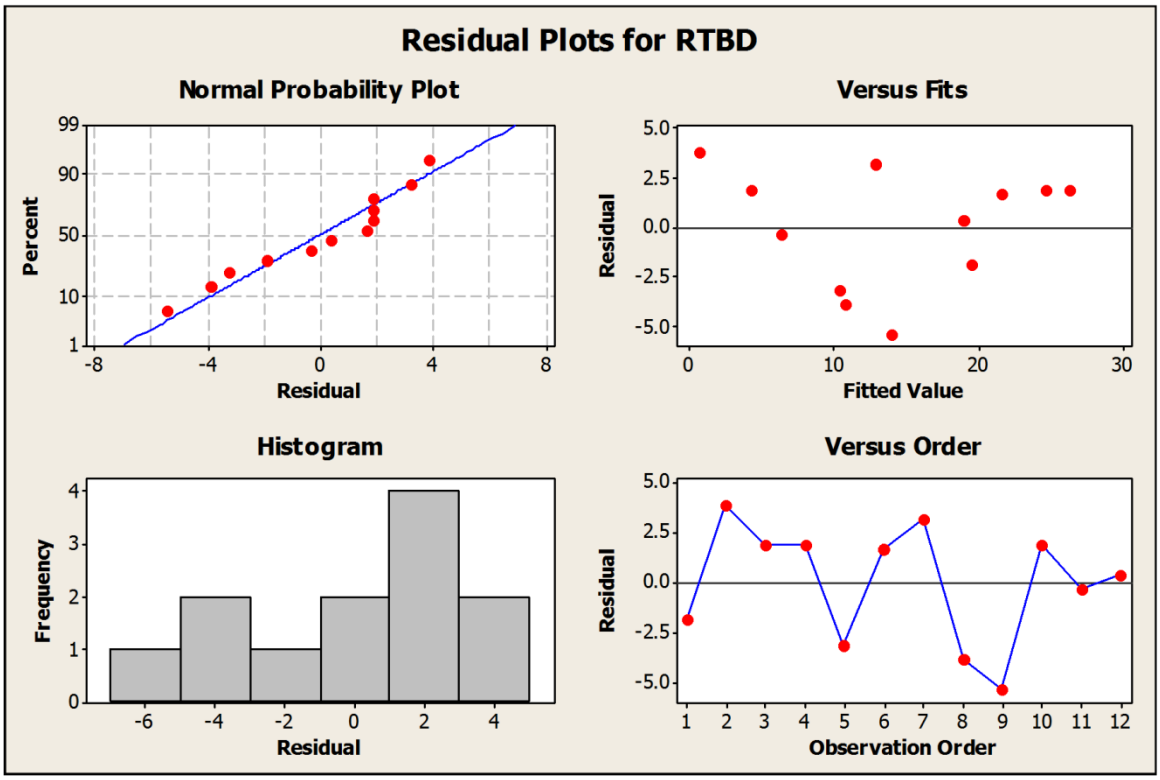


Figure 3.8 Residual Plots for RTBD

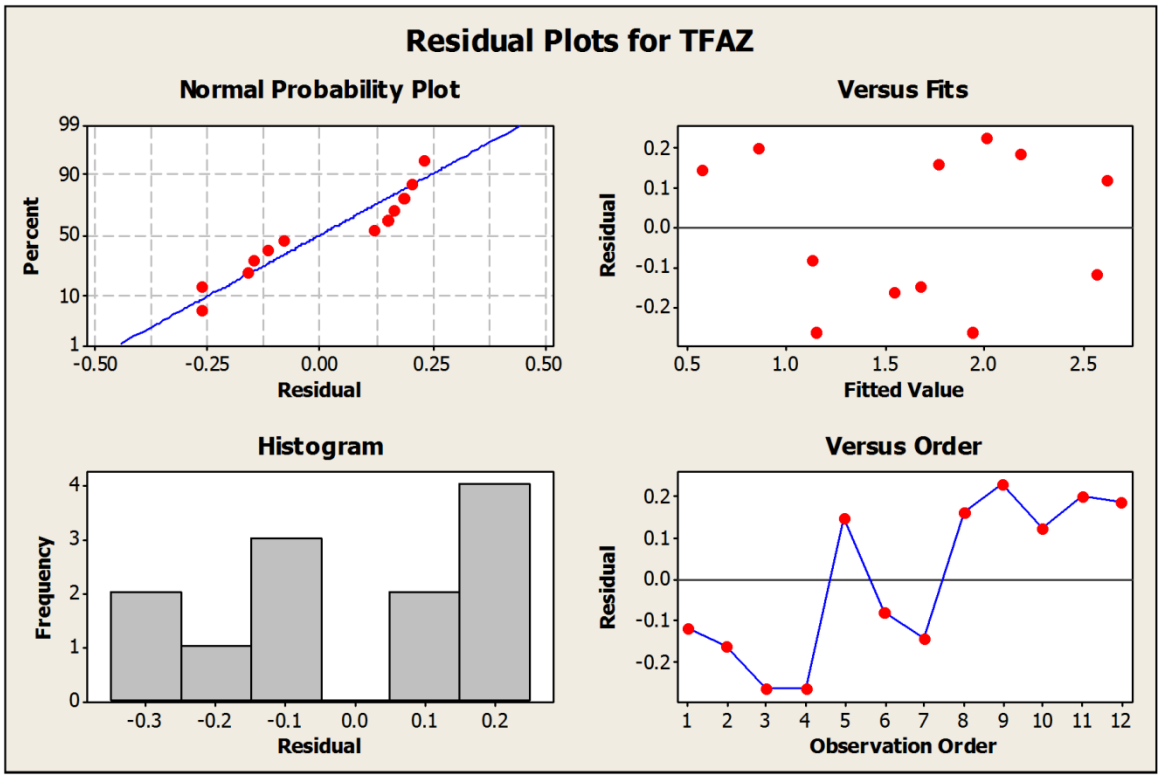


Figure 3.9 Residual Plots for TFAZ

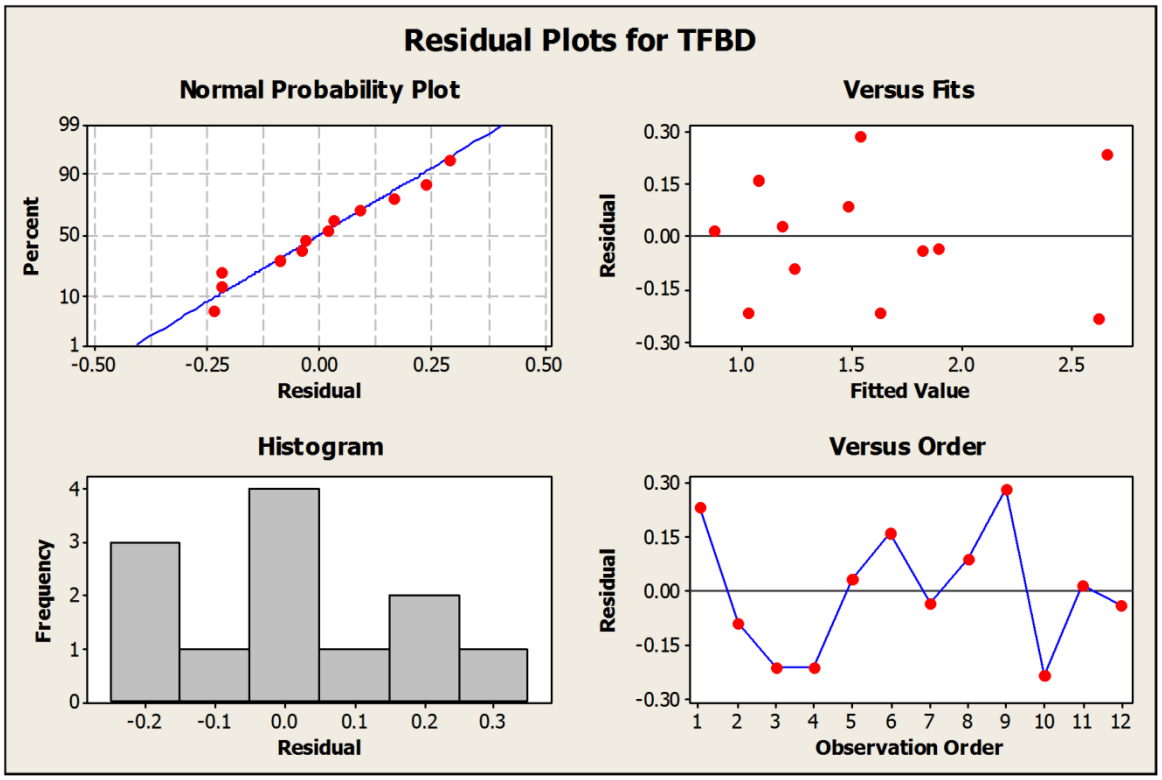


Figure 3.10 Residual Plots for TFBD

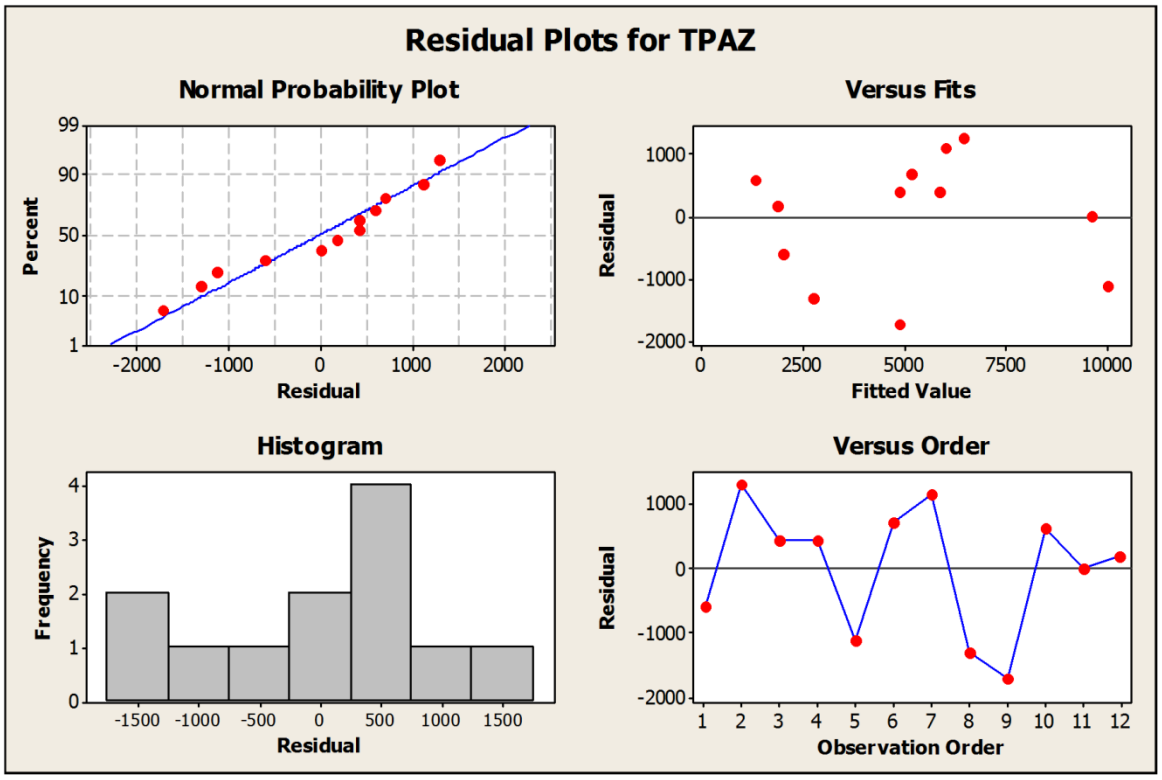


Figure 3.11 Residual Plots for TPAZ

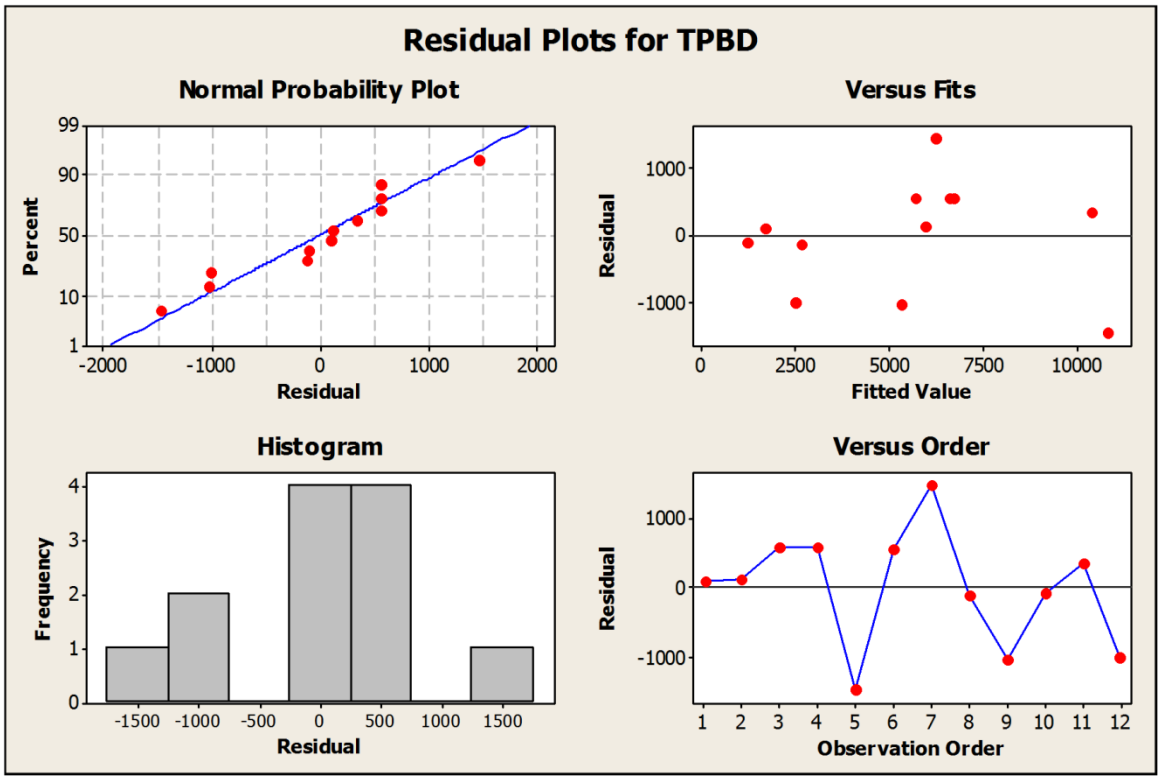


Figure 3.12 Residual Plots for TPBD

The residual plots define that the normal distribution of data, the residuals are scattered randomly as seen in the plots of residual versus fitted and the plots of residual versus order of data show that the order of data does not significantly affect the results.

3.5.2.3. The statistical outputs of Plackett-Burman design

The statistical (factorial) fit for the generated PBD model has been given in Table 3.5. The proportion of variation in the response data explained by the terms in the model, is determined by R^2 and adjusted R^2 values. R^2 is the proportion of the variability in the response that is explained by the model. Adjusted R^2 is a modified version of R^2that adjusts for the number of terms in the model.

Table 3.5 Factorial fit for the developed model

RESPONSE	SD	R^2	R^2(adjusted)
RS	4.00	82.09%	70.76%
TFAZ	0.31	92.02%	88.07%
TFBD	0.29	91.80%	77.44%
TPAZ	1618.2	89.40%	70.85%
TPBD	1367.9	93.44%	81.96%
RTAZ	1.74	88.98%	79.70%
RTBD	4.93	88.27%	76.75%

The estimated coefficients of the responses are given in Table 3.6 which define the magnitude of each factor on each response and its influence (in terms of positive of negative). Accordingly, the factor, Org Conc. influences TPAZ and TPBD positively and the factors, Temp and pH effect negatively on the responses TPAZ and TPBD. Similarly the inferences of other factors can be obtained.

The p-values for the responses with respect to various factors is given in Table 3.7. The factors whose p-value is less than 0.05 indicate that they have a significant effect. The p-value for flow rate was higher in most of the cases but according to the half-normal and pareto charts, the flow-rate was shown as one of the significant factor affecting the retention time of AZ and BD. Hence the flow-rate was taken as one of the three factors for the further optimization of HPLC method by using Box-Behnken

design. Thus pH, FR and Org Conc. were found to be significant and were selected for further optimisation.

Table 3.6 Estimated coefficients for the Responses

TERMS	COEFFICIENTS						
	RS	RTAZ	RTBD	TFAZ	TFBD	TPAZ	TPBD
CONSTANT	1.16	0.50	1.43	0.09	0.08	467.1	394.9
BUFF	1.14	-0.20	1.06	0.12	0.07	-208.0	-431.0
COL	3.40	-0.72	-1.04	0.12	0.26	-311.0	-725.0
TEMP	-1.68	-0.27	-0.27	0.05	-0.09	-602.0	-757.0
pH	2.75	0.29	1.52	0.50	0.44	-1417.0	-1543.0
FR	-0.27	-1.53	-4.10	0.14	0.03	-302.0	-508.0
ORG CONC.	-0.85	-2.25	-6.11	-0.23	-0.19	2077.0	2102.0
BUFF CONC.	0.68	0.13	1.55	-0.17	-0.01	668.0	739.0

Table 3.7 Estimated p-values for the Responses

TERMS	p-values						
	RS	RTAZ	RTBD	TFAZ	TFBD	TPAZ	TPBD
BUFF	0.38	0.71	0.50	0.25	0.43	0.68	0.34
COL	0.04	0.22	0.51	0.25	0.04	0.54	0.14
TEMP.	0.22	0.62	0.86	0.60	0.36	0.27	0.13
pH	0.08	0.60	0.35	0.01	0.01	0.04	0.02
FR	0.83	0.04	0.05	0.19	0.75	0.55	0.27
ORG CONC.	0.50	0.01	0.01	0.06	0.08	0.01	0.01
BUFF CONC.	0.59	0.80	0.34	0.14	0.90	0.23	0.14

3.5.3. Conclusion from the Plackett-Burman design

Three factors i.e. pH, organic ratio and flow rate were found to be the most significantly affecting (almost all) the responses involved. Hence, these 3 factors were selected for the next stage of optimization in QbD. The trials with phosphate buffer showed comparatively asymmetric peaks than the ammonium formate buffer. Hence the latter was selected as the final buffer for method development. Desired results i.e. SST parameters were obtained on C8 column as compared to C18 column hence C8 column was chosen for the analysis.

3.6. BOX-BEHNKEN DESIGN FOR RP-HPLC METHOD OPTIMISATION AND VALIDATION OF DEVELOPED RP-HPLC METHOD

3.6.1. EXPERIMENTAL

3.6.1.1. The Box-Behnken Design

Based on the three selected factors obtained from Plackett-Burman screening design i.e. pH, percentage organic concentration (OC) and flow rate (FR), these were now used to get an optimized method which led to the generation of Design space in which the method was found to be robust. Box-behnken design of experiments approach was employed to generate an optimized method. The factors and their levels are shown in Table 3.8.

Table 3.8 Factors and their levels for experimental design

Factors	Factor ID	Low	middle	high
pH	A	3	4	5
OC	B	40	50	60
FR	C	0.8	1	1.2

3.6.1.2. Preparation of standard solutions

AZ stock solution (1 mg/mL): Accurately weighed 25 mg AZ was transferred in 25 mL volumetric flask, dissolved and then diluted with MeOH up to the mark.
AZ working solution (0.856 mg/mL): The working solution was prepared by transferring 21.4 mL from of AZ stock solution to 25 mL volumetric flask and diluted to the mark with mixture of mobile phase (ammonium formate buffer: ACN =50:50).

BD stock solution (0.5 mg/mL): Accurately weighed 12.5 mg BD was transferred in 25 mL volumetric flask, dissolved and then diluted with MeOH up to the mark.
BD working solution (0.2 mg/mL): The working solution was prepared by transferring 10 mL from of BD stock solution to 25 mL volumetric flask and diluted to the mark with mixture of mobile phase (ammonium formate buffer: ACN =50:50).

3.6.1.3. Preparation of sample solution

1mL of laboratory mixture was taken in 10 mL volumetric flask dissolved in minimum amount of MeOH, made upto the mark with the mobile phase (50:50 mixture of buffer and ACN). The solution was filtered with 0.2 µ membrane filter and analysed by RP-HPLC.

3.6.1.4. RP-HPLC method validation

The validation of the RP-HPLC method was carried out in accordance with the ICH guidelines (22). The method was validated for various parameters like linearity, accuracy, precision, limit of detection, limit of quantification, sensitivity, selectivity and robustness.

The linearity of the RP-HPLC detector response for determination of AZ and BD was evaluated by analysing a series of different concentrations of each compound. The calibration range was established with respect to the practical range (according to content and ratio of each compound with respect to the dose) necessary, to give accurate, precise and linear results. Seven concentrations were chosen, ranging from 4.28-342.4 µg/mL AZ, and 1-80 µg/mL BD and the linearity was determined. Mixed standard solutions of AZ and BD were prepared in ratio of 4.28:1 as present in the laboratory mixture (with reference to the doses).

For evaluation of the precision estimates, intra-day and inter-day precision were performed at three concentration levels in triplicates. The concentration levels were: 4.28, 85.6, 342.2 µg/mL for AZ and 1, 20, 80 µg/mL for BD. The peak areas of the two drugs were calculated for each trial. The experiment was repeated three times in a day for intra-day precision and on three different days for inter-day precision.

Accuracy was determined by standard addition method at three levels of standard addition i.e. 80%, 100%, and 120%. The concentration levels (0, 80, 100, 120 %) for accuracy study were: 85.6, 154.08, 171.2, 188.32 µg/mL and 20, 36, 40, 44 µg/mL for BD. The resulting mixtures were analysed and results obtained were compared with the expected results.

According to ICH recommendations (22), the approach based on the standard deviation (S.D.) of the y-intercept and the slope was used for determining the limit of detection (LOD) and limit of quantitation (LOQ).

Various factors were assessed to check the robustness of the method. The factors such as: pH (2.8, 3.0, 3.2), flow rate (0.8, 1, 1.2) and percentage organic concentration (OC) (58, 60, 62) were varied in the region of design space, generated by applying QbD in method optimization and the robustness of the method was determined.

The standard stock solutions prepared in the MeOH were kept for 24 h when kept at room temperature and for 48 h when stored in refrigerator (8-25 ºC) and the stability of stock solutions was determined.

According to ICH recommendation, the method is specific when the results are unaffected by the presence of the dosage form excipients. The specificity of the method was determined by analysing the laboratory mixture to check any interference of excipients.

System suitability parameters such as theoretical plates, symmetry factor and resolution for AZ and BD were calculated for n=6 replicates to study the system suitability of RP-HPLC method.

3.6.1.5. Analysis of laboratory mixture

The RP-HPLC method was successfully applied to the determination of AZ and BD in laboratory mixture without the interference of excipients therein. 5 mL of laboratory mixture was taken in 100 mL volumetric flask dissolved in minimum amount of MeOH and was made upto the mark with the mobile phase (50:50 mixture of buffer and ACN). The solution was filtered with 0.2 µ membrane filter and analysed by RP-HPLC.

3.6.2. RESULT AND DISCUSSION

The BBD including 14 runs was worked out for three factors and the results of runs are shown in Table 3.9. Few trials have been shown in Figure 3.13. In analysis window the model were chosen on the basis of ANOVA results which showed significant p-value, R^2 value and F value. It also showed reasonably good agreement

between adjusted and predicted R^2 value. The model equations of various responses have been shown in Table 3.10 and the ANOVA results along with the model summary statistics are given in Table 3.11. The contour (2D) plots of responses with respect to all factors are shown in Figure 3.14 to 3.20 for RS, RTAZ, RTBD, TFAZ, TFBD, TPAZ and TPBD respectively. The optimum conditions were calculated using numerical optimization. To achieve the composite desirability (D), the response criteria were set as (lower–upper): TFAZ as (0.5-1.2), TFBD as (0.5-1.2), TPAZ as (>3000), TPBD as (>3000) and RS as (>2). The Derringer's desirability was calculated for the set criterions, which indicated that maximum desirability was achieved at 0.97.

Table 3.9 BBD trials and responses

Trial	**Factors**			**Responses**						
No.	pH	OC	FR	RTAZ	RTBD	TPAZ	TPBD	TFAZ	TFBD	RS
1	4	60	0.8	5.43	8.68	4457.8	4268.3	2.11	1.67	4.21
2	4	40	1.2	8.90	23.37	3932.3	4070.4	1.35	0.72	7.20
3	4	50	1	5.74	11.72	3974.8	3541.0	1.63	1.02	4.35
4	3	50	1.2	4.52	10.09	4183.2	3881.4	1.69	0.97	5.91
5	5	60	1	7.00	7.00	3516.9	3516.9	1.21	1.21	0.00
6	3	60	1	4.13	7.16	5582.2	6476.4	1.01	1.29	5.24
7	4	40	0.8	12.90	33.49	3407.6	3164.4	1.10	0.80	3.17
8	4	60	1.2	3.69	5.89	4624.9	4726.7	2.34	1.80	4.70
9	3	40	1	9.61	26.23	3458.2	3108.9	0.99	0.83	3.75
10	5	40	1	14.45	26.39	3982.3	3444.4	1.08	0.90	3.49
11	5	50	1.2	7.22	9.76	3729.8	4290.3	2.34	0.86	2.35
12	4	50	1	5.84	11.96	3939.8	3575.0	1.60	0.97	4.44
13	3	50	0.8	6.61	14.15	3800.8	3391.8	1.45	1.12	3.98
14	5	50	0.8	10.47	14.40	3620.0	3540.9	1.56	1.02	1.89

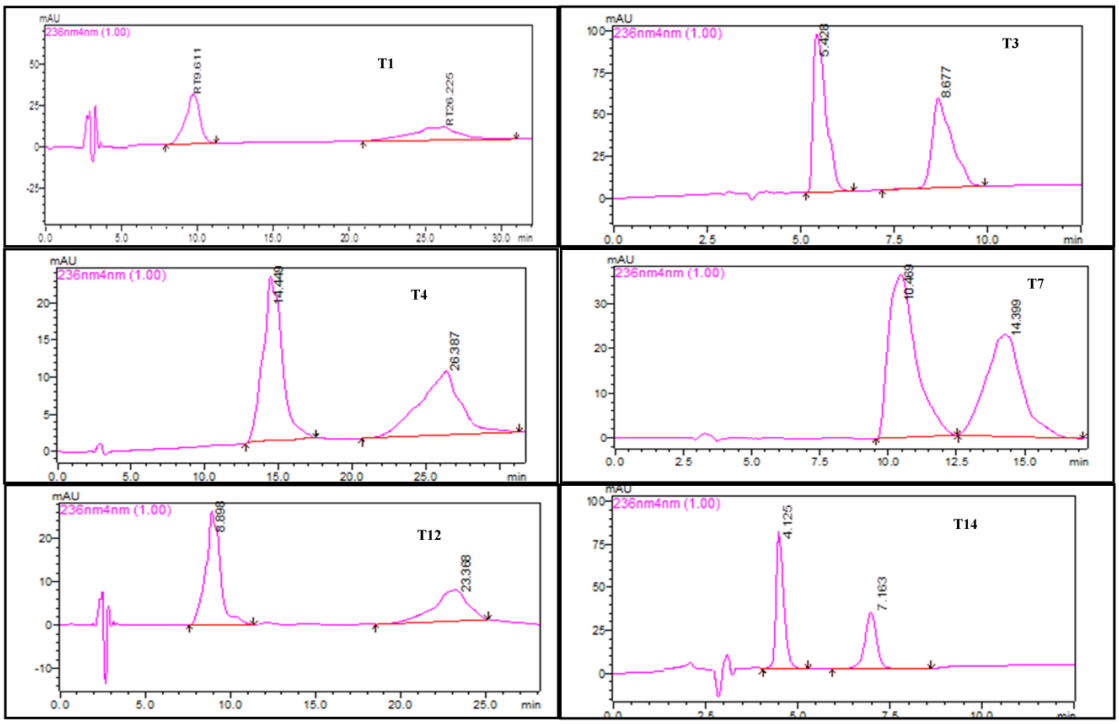


Figure 3.13 Chromatograms of few trials of Box-Behnken Design

(1st peak AZ, 2nd peak BD)

Table 3.10 Model Equations (in terms of coded values)

Responses	Equations
RS	RS = 4.39 - 1.39A - 0.43B + 0.86C - 1.24AB - 0.37AC - 0.88BC - $1.28A^2$ + $0.016B^2$ + $0.42C^2$
RTAZ	RTAZ = 5.79 + 1.78A - 3.20B - 1.38C - 0.49AB -0.28AC + 0.56BC + $1.24A^2$ + $1.76B^2$ + $0.17C^2$
RTBD	RTBD = 11.84 - 0.011A - 10.09B - 2.70C - 0.081AB - 0.14AC + 1.83BC - $0.45A^2$ + $5.30B^2$ + $0.71C^2$
TFAZ	TFAZ = 1.61 + 0.13A + 0.27B + 0.19C + 0.03AB + 0.14AC + 0.0045BC - $0.25A^2$ – $0.29B^2$ + $0.40C^2$
TFBD	TFBD = 1 - 0.03A + 0.34B - 0.03C - 0.04AB + 0.0028AC + 0.05BC - $0.1A^2$ - $0.16B^2$ + $0.09C^2$
TPAZ	TPAZ = 3957.3 - 271.9A - 425.2B + 148.0C -647.3AB -68.2AC -89.4BC - $47.3A^2$ + $224.9B^2$ - $76.5C^2$
TPBD	TPBD = 3558.0 - 258.2A - 650.0B + 325.4C - 823.7AB + 64.9AC 111.9BC + $148.6A^2$ + $430.0B^2$ + $69.4\ C^2$

Equations in terms of coded values [A=pH, B=OC, C=FR]

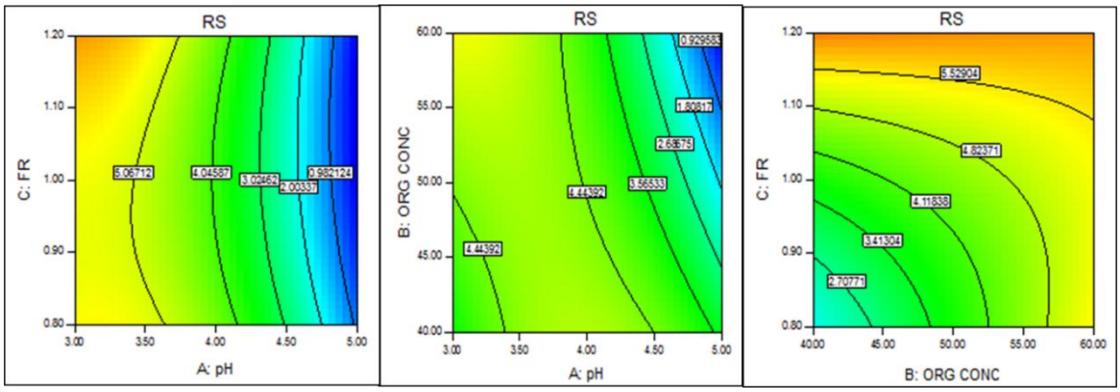


Figure 3.14 2D contour plots of response RS with respect to all the three factors

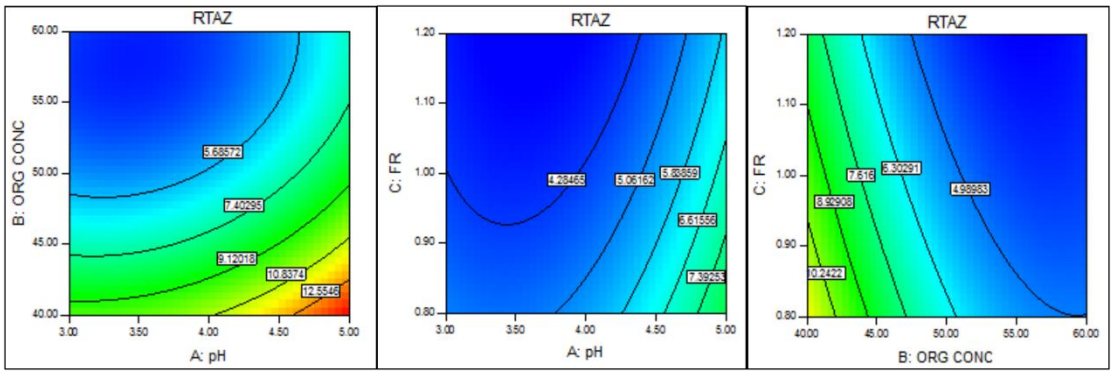


Figure 3.15 2D contour plots of response RTAZ with respect to all the three factors

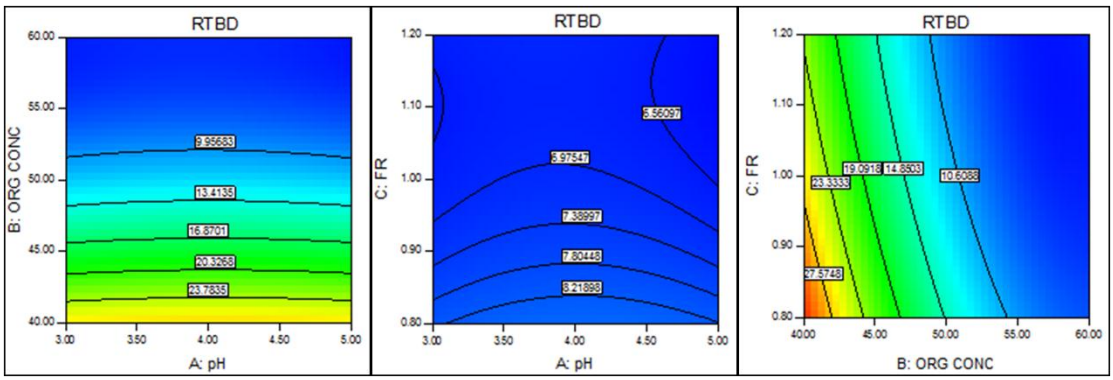


Figure 3.16 2D contour plots of response RTBD with respect to all the three factors

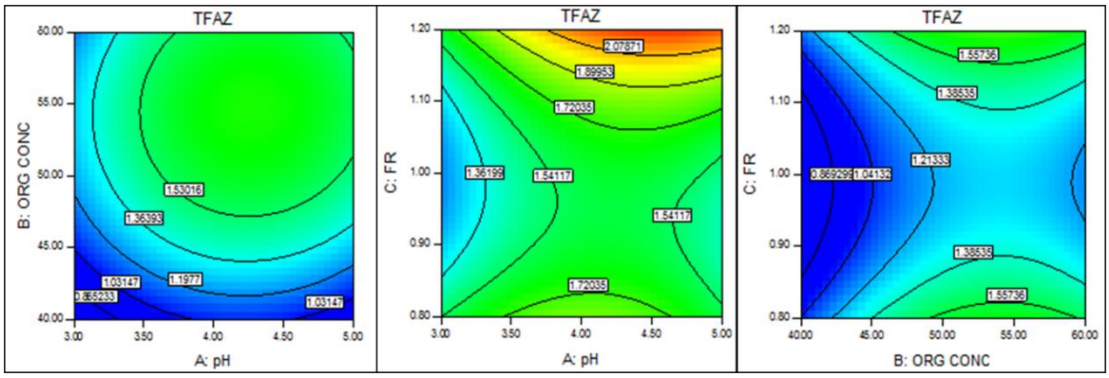


Figure 3.17 2D contour plots of response TFAZ with respect to all the three factors

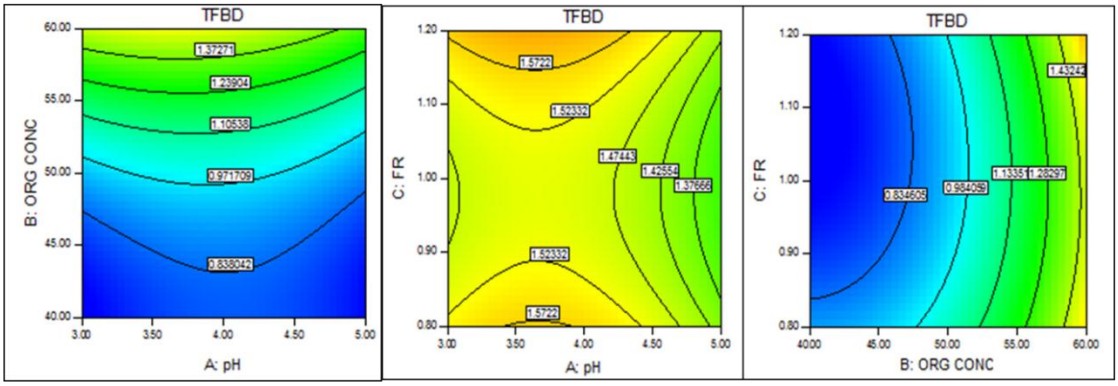


Figure 3.18 2D contour plots of response TFBD with respect to all the three factors

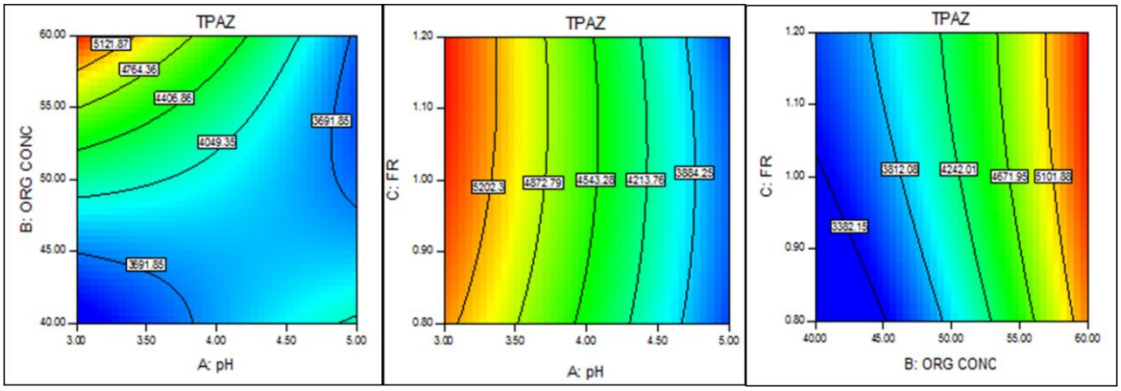


Figure 3.19 2D contour plots of response TPAZ with respect to all the three factors

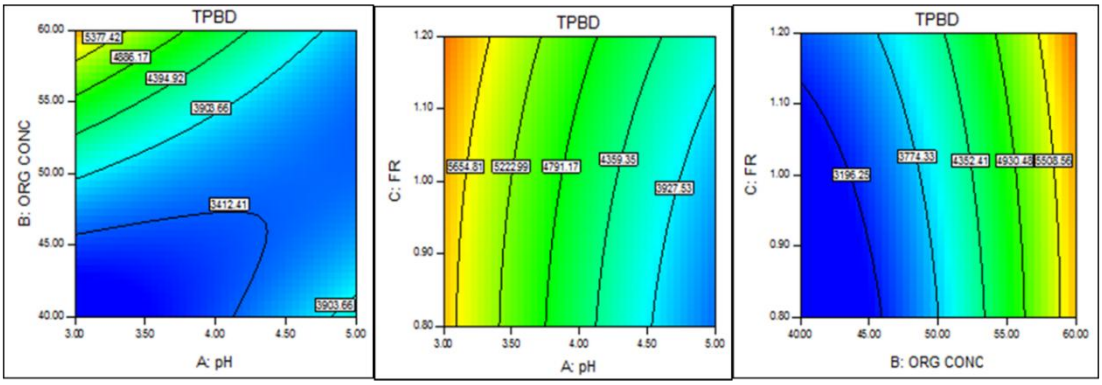


Figure 3.20 2D contour plots of response TPBD with respect to all the three factors

Table 3.11 Model Summary statistics

Statistical parameters	Responses						
	RS	**RTAZ**	**RTBD**	**TFAZ**	**TFBD**	**TPAZ**	**TPBD**
SD	0.39	0.22	1.01	0.35	0.22	165.29	636.57
Mean	3.90	7.61	15.02	1.53	1.08	4015.04	3928.35
CV %	10.00	2.94	6.73	22.93	20.44	4.12	16.20
R^2	0.98	1.00	1.00	0.82	0.85	0.97	0.83
Adj R^2	0.95	1.00	0.99	0.83	0.79	0.92	0.86
Pred R^2	0.76	0.98	0.93	0.74	0.73	0.89	0.78
F-value	28.94	307.57	107.12	16.95	2.23	2.05	2.51
p value	0.0027	0.019	0.0002	0.0076	0.023	0.025	0.019

3.6.2.1. Robustness of the DoE model

To check the robustness of the model, five solutions (as stated in Table 3.12) among the generated solutions (23 solutions) were selected and chromatographed. To check the point prediction the experimental values were compared with the predicted values of responses. It was found that the experimental values lie within 95% confidence and predicted intervals (shown in Table 3.12). Finally solution with pH 3, organic concentration as 60% and flow rate 1mL/min, was chosen to record the chromatogram which allowed the complete separation of the two compounds under study. The chromatogram of the standard solution mixture of AZ and BD is shown in Figure 3.21.

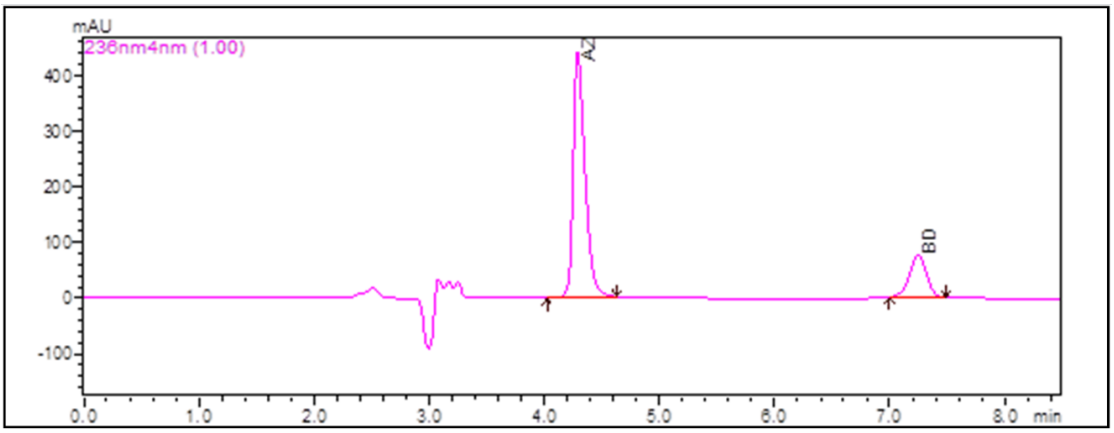


Figure 3.21 Chromatogram of standard solution mixture containing 85.6 ppm of AZ and 20 ppm of BD

Table 3.12 Factors and targeted criteria used in Design expert

Sr. No.	pH	%OC	FR	Response	PV	EV	95% CI Predicted		95% PI Predicted	
							low	High	Low	high
1.	3	60	1.17	RS	5.90	5.56	4.73	7.06	4.31	7.48
				RTAZ	3.98	4.21	3.32	4.65	3.07	4.89
				RTBD	6.58	7.129	3.57	9.59	2.46	10.69
				TFAZ	1.50	1.4	0.45	2.54	0.06	2.92
				TFBD	1.55	1.6	0.89	2.21	0.65	2.45
				TPAZ	5531.85	5195.75	5039.48	6023.94	4858.73	6204.69
				TPBD	6040.51	5702.54	4142.72	7934.00	3446.63	8630.10
2.	3	60	1.08	RS	5.51	5.89	4.74	7.06	4.31	7.49
				RTAZ	4.13	4.19	3.30	4.63	3.06	4.87
				RTBD	6.52	6.915	3.59	9.59	2.48	10.70
				TFAZ	1.26	1.23	0.47	2.55	0.08	2.94
				TFBD	1.49	1.47	0.89	2.21	0.65	2.45
				TPAZ	5516.23	6104	5029.56	6011.50	4848.47	6192.59
				TPBD	5934.40	5637.63	4134.04	7915.64	3436.64	8613.04

				RS	5.31	5.9	4.46	6.36	3.97	6.85
				RTAZ	4.32	4.1	3.66	4.75	3.38	5.03
				RTBD	6.73	7.2	4.12	9.03	2.85	10.30
3.	3	60	0.99	TFAZ	1.18	1.084	0.36	2.06	-0.09	2.51
				TFBD	1.46	1.42	0.94	2.01	0.66	2.29
				TPAZ	5473.88	5986.45	5100.05	5903.12	4891.80	6111.38
				TPBD	5862.51	5927.63	4354.71	7447.45	3552.69	8249.46
				RS	6.09	6.42	4.26	6.24	3.78	6.72
				RTAZ	3.85	3.72	4.00	5.13	3.72	5.40
				RTBD	6.51	6.81	4.58	9.70	3.34	10.93
4.	3	59.17	1.2	TFAZ	1.65	1.73	0.35	2.13	-0.08	2.56
				TFBD	1.52	1.67	0.91	2.03	0.64	2.31
				TPAZ	5412.46	5823.64	4992.20	5828.70	4789.53	6031.37
				TPBD	5905.59	5636.61	4206.77	7428.26	3426.25	8208.79
				RS	5.39	6.15	4.21	6.33	3.76	6.79
				RTAZ	5.00	4.52	4.10	5.32	3.84	5.58
				RTBD	8.13	7.4	4.71	10.19	3.53	11.37
5.	3	59.95	0.8	TFAZ	1.54	1.63	0.36	2.27	-0.05	2.68
				TFBD	1.53	1.58	0.89	2.09	0.63	2.35
				TPAZ	5267.30	5714.56	4917.77	5815.56	4724.70	6008.63
				TPBD	5776.17	5638.64	4073.20	7530.72	3329.66	8274.26

*Solution (Soln) used as the final optimised chromatographic condition
PV=predicted value; EV=experimental value

3.6.2.2. Validation of the DoE model

To validate the model, six check points were selected and the results of the experimental values obtained were compared with the predicted values. The results suggested quite resemblance between the experimental and model generated values resulting in low residual values presented in terms of percentage bias (% bias) (Table 3.13). Figure 3.22 shows the 3D desirability contour plots and Figure 3.23 shows the design space for all the parameters. The final optimised RP-HPLC parameters are shown in Table 3.14.

Table 3.13 Results for validation of model (check point trials)

Trial No.	Factors			Responses	Experimental Value	Predicted value	% Bias
	pH	OC	FR				
1	3.5	40	1	RS	4.59	4.24	7.53
				RTAZ	9.93	10.10	1.73
				RTBD	27.09	26.40	2.55
				TFAZ	3402.16	3275.45	3.72
				TFBD	2995.58	2846.23	4.99
				TPAZ	0.94	0.85	9.54
				TPBD	0.76	0.72	5.13
2	3.5	40	1.2	RS	6.94	7.14	2.93
				RTAZ	8.30	8.23	0.82
				RTBD	23.34	23.75	1.77
				TFAZ	3673.67	3473.65	5.44
				TFBD	3400.46	3587.92	5.51
				TPAZ	1.46	1.52	4.06
				TPBD	0.73	0.68	6.28
3	4.5	40	0.8	RS	3.29	3.12	5.13
				RTAZ	14.47	14.76	2.00
				RTBD	32.48	35.14	8.20
				TFAZ	3574.23	3792.73	6.11
				TFBD	3091.25	3184.73	3.02
				TPAZ	1.18	1.09	7.84
				TPBD	0.76	0.84	9.81
4	3.5	60	0.8	RS	5.22	5.64	7.99
				RTAZ	4.86	5.25	7.93
				RTBD	8.49	9.23	8.68
				TFAZ	4807.17	4827.54	0.42
				TFBD	4938.36	5013.27	1.52
				TPAZ	1.74	1.64	5.49
				TPBD	1.47	1.53	4.26

				RS	2.33	1.98	14.93
				RTAZ	5.31	5.73	7.81
				RTBD	6.89	7.42	7.64
5	4.5	60	1	TFAZ	3980.59	4073.65	2.34
				TFBD	4037.38	4275.74	5.90
				TPAZ	1.61	1.74	8.26
				TPBD	1.41	1.53	8.46
				RS	2.54	2.31	9.21
				RTAZ	4.52	4.73	4.58
				RTBD	6.66	6.14	7.83
6	4.5	60	1.2	TFAZ	4005.11	3802.65	5.05
				TFBD	4283.34	4143.64	3.26
				TPAZ	2.26	2.17	3.82
				TPBD	1.38	1.49	7.87

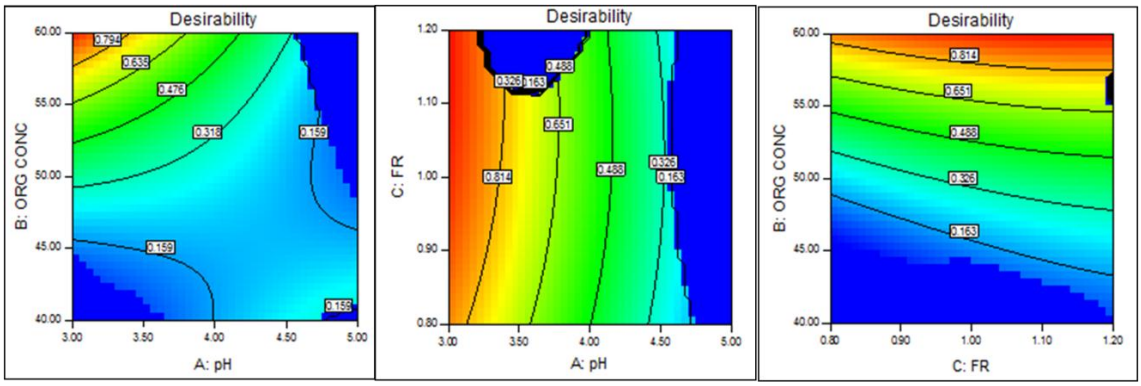


Figure 3.22 Desirability 3D plots for the three factors for optimised method

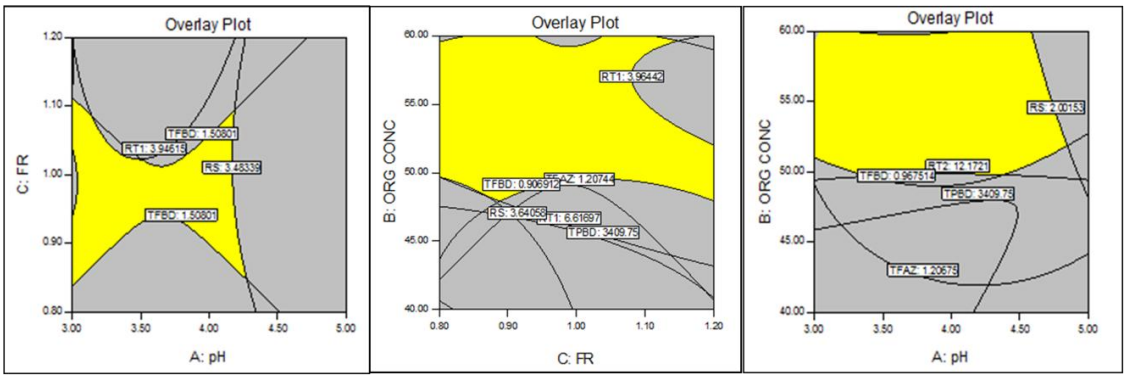


Figure 3.23 Plots for the design space

Table 3.14 Final optimised RP-HPLC parameters

Method parameters	Optimised value	
Column	Oyster C8 column (250 mm × 4.6mm i.d × 5µm particle size)	
Mobile phase	ACN: 10 mM Ammonium formate buffer (pH 3) = 60:40	
Flow rate	1.0 mL/min	
Temperature	Ambient	
Detection wavelength	236 nm	
Retention Time (min)	AZ	4.15
	BD	7.26
Needle wash	Mobile phase	

3.6.2.3. Validation of RP-HPLC method

3.6.2.3.1. Linearity

Characteristic parameters for regression equations of the RP-HPLC method are given in Table 3.15. The regression coefficients for AZ and BD were found to be 0.9990 and 0.9991 respectively. The overlain chromatograms and calibration curve plots are shown in Figure 3.24 and 3.25 respectively.

Table 3.15 Statistical results of calibration

Parameters	AZ	BD
Calibration range (µg/mL)	4.28-342.4	1-80
Regression equation y=mx+c	y = 36380x + 83363	y = 45128x - 18017
Correlation coefficient(r^2)	0.9990	0.9991

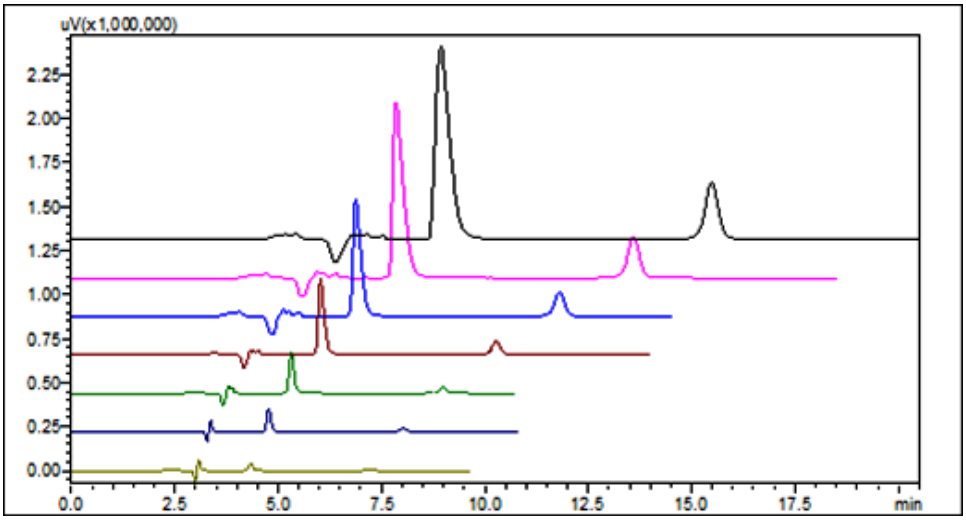


Figure 3.24 Overlain chromatograms of the standard solutions (1st peak of AZ and 2nd peak of BD)

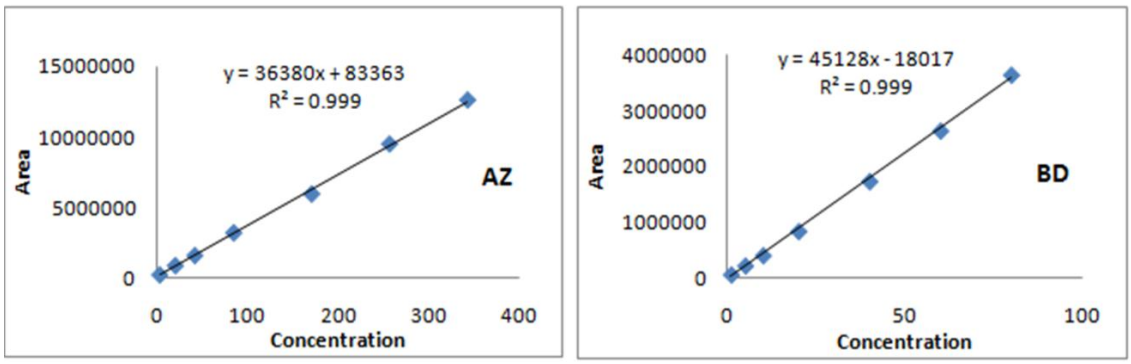


Figure 3.25 Calibration curve plots of AZ and BD [Peak area vs Conc. (µg/mL)]

3.6.2.3.2. Precision

The RP-HPLC method was found to be quite precise. The precision expressed as the average percentage relative standard deviation (% RSD) for intra-day and inter-day measurements for AZ and BD is given in Table 3.16 and 3.17 respectively.

Table 3.16 Intra-day precision studies

DRUG CONC.	PEAK AREA			%RSD	MEAN %RSD
			AZ		
4.28	278450.7	282384.3	284683.7	1.12	
85.6	3321661	3371903	3360410	0.79	
342.4	12644906	12881369	12612077	1.16	
					1.02
			BD		
1	75154.33	75535	75845.33	75511.56	
20	847397	853144.7	854981.7	851841.1	
80	3689197	3678653	3780286	3716045	
					0.81

Table 3.17 Inter-day precision studies

DRUG CONC.	PEAK AREA			%RSD	MEAN %RSD
	*DAY1	*DAY2	*DAY3		
			AZ		
4.28	298450.7	305524.7	306473.7	1.44	
85.6	3321661	3238818	3331478	1.54	
342.4	12644906	12964485	12825597	1.25	
					1.41
			BD		
1	77284.67	76517.67	75096.67	76299.67	
20	857923	867727.7	868027	864559.2	
80	3735046	3779234	3699417	3737899	
					1.06

3.6.2.3.3. Accuracy

The excellent recoveries of standard addition method (Table 3.18) for RP-HPLC suggested good accuracy of the proposed method.

Table 3.18 Recovery results from laboratory mixture

	Percentage ± standard addition			
% Addition	0	80	100	120
		AZ		
Theoretical Content (µg/mL)	85.6	154.08	171.2	188.32
Amount Found (µg/mL)	152.56	152.56	169.95	188.03
Recovery (%) ± S.D.	100.75± 0.24	99.02±0.42	99.23±6 0.44	99.85± 0.29
		BD		
Theoretical Content (µg/mL)	20	36	40	44
Amount Found (µg/mL)	19.84	35.63	39.51	44.13
Recovery (%) ± S.D.	99.20±1.0	98.96±0.93	98.78±0.81	100.30±1.01

3.6.2.3.4. Detection and quantitation limits

The limit of detection (LOD) and limit of quantitation (LOQ) and values thus found are given in Table 3.19.

Table 3.19 Results of LOD and LOQ

Parameters	AZ	BD
LOD (µg/mL)	0.16	0.14
LOQ (µg/mL)	0.49	0.43

3.6.2.3.5. Robustness

The results of robustness for pH, flow rate and OC are shown in Table 3.20. The RP-HPLC method was found to be robust at various levels of robustness for various factors.

Table 3.20 Robustness results

Parameters	**Levels**	**AZ**	**BD**
pH	2.8	4.2	7.23
	3.0	4.20	7.24
	3.2	4.22	7.25
	Mean	4.21	7.24
	SD	0.013	0.010
FR	0.8	4.29	7.18
	1	4.20	7.24
	1.2	4.19	7.14
	Mean	4.22	7.19
	SD	0.05	0.05
OC	23	4.41	7.69
	24	4.20	7.24
	25	3.95	7.16
	Mean	4.18	7.36
	SD	0.23	0.28

3.6.2.3.6. Stability

The standard solutions prepared in the mobile phase exhibited no chromatographic or absorbance changes for 12 h when kept at room temperature and for 24 h when stored in refrigerator (8-25 ºC). No additional peak was found and no significant change in the peak area was observed which indicated the stability of the standard solutions under study.

3.6.2.3.7. Specificity

The method was found to be specific as no interference of excipients was found. No additional peak of any excipient as found in the chromatogram of the sample solution (Figure 3.26).

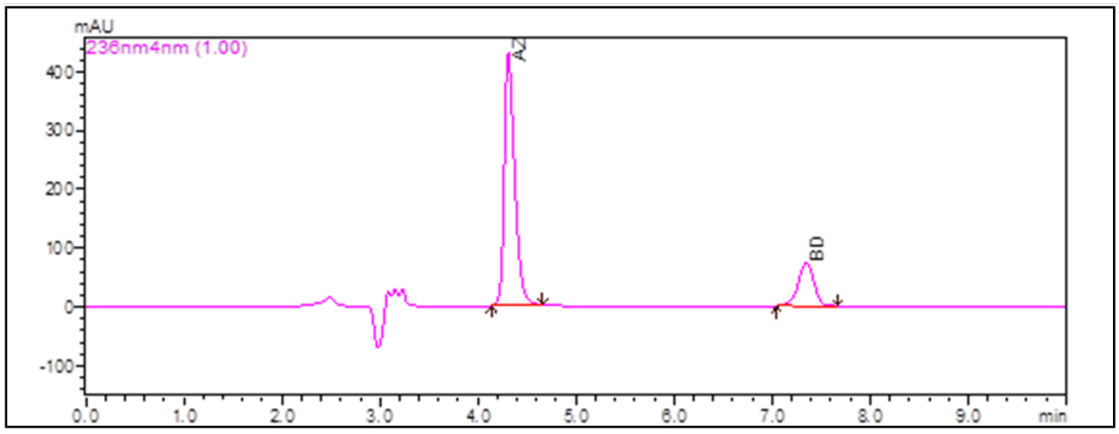


Figure 3.26 Chromatogram of sample solution mixture containing 85.6 ppm of AZ and 20 ppm of BD

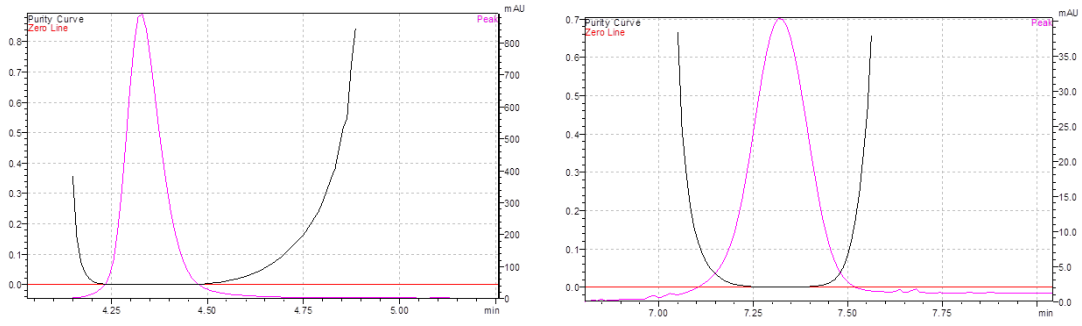
3.6.2.3.8. System suitability

Satisfactory results were obtained for system suitability as shown in Table 3.21. The peak purity curves have been shown in Figure 3.27 and the peak purity results are given in Table 3.22.

Table 3.21 System Suitability Parameters for the developed RP-HPLC method

Parameters	AZ	BD
Retention Time	4.15 ± 0.013	7.26 ± 0.045
Tailing factor	1.61 ± 0.03	0.91 ± 0.044
Resolution	--	11.76 ± 0.56
Theoretical Plates	6845.77 ± 1.37	9067.3 ± 1.52

Mean±standard deviation for n=6 replicates



(AZ) **(BD)**

Figure 3.27 The peak purity curves of AZ and BD

Table 3.22 Peak Purity results

Drug Name	Peak Purity Index	Threshold
AZ	1.0000	0.9999
BD	0.9999	0.9989

3.6.2.4. Summary of validation parameters

The results of the RP-HPLC method validation have been summarised in Table 3.23.

Table 3.23 Summary of validation parameters

Parameters	AZ	BD
Calibration range (µg/mL)	4.28-428	1-80
LOD (µg/mL)	0.16	0.14
LOQ (µg/mL)	0.49	0.43
Regression equation y=mx+c	y = 36380x + 83363	y = 45128x - 18017
Correlation coefficient (r^2)	0.9990	0.9991
80%	99.02±0.42	98.96±0.93
100%	99.23±6 0.44	98.78±0.81
120%	99.85± 0.29	100.30±1.01
Intraday	1.02	0.81
Interday	1.09	1.06

3.6.2.5. Analysis of laboratory mixture

The RP-HPLC method was successfully applied to the determination of AZ and BD in the laboratory mixture without the interference of excipients therein. The results of the assay are shown in Table 3.24.

Table 3.24 Results for assay of laboratory mixture

	AZ	**BD**
Content in lab mixture (%w/v)	85.6	20
% Assay±SD	99.89±1.07	100.07±1.48

Determination for n=6 replicates

3.7. CONCLUSION

The Design of experiment was successfully applied for the optimisation and development of RP-HPLC method for the simultaneous estimation of Azelastine hydrochloride and Budesonide, wherein Plackett-Burman design was used for the screening of major factors. Three major factors as found by PBD were further optimised by Box-Behnken design and thus an optimised RP-HPLC method was developed for the estimation of AZ and BD giving best separation of the two components within shortest possible time period and appropriate SST parameters. The method was found to be LC-MS compatible, it was transferred to LC-MS analysis and a bioanalytical method was developed for the estimation of AZ and BD in human plasma.

3.8. REFERENCES

1. http://www.empr.com/phase2studyofbudesonideazelastinenasalsprayforseasonalallergicrhinitis/article/128869/ Accessed on: 29 June 2015.
2. Salapatek A, D'Angelo P, Bates M, Patel D, Patel P, Pipkin J, Zimmerer R. Novel Combination Product Captisol-Enabled Budesonide + Azelastine HCl Provides Fast, Long-lasting Relief of Allergic Rhinitis Symptoms (Total Nasal Symptom Score (TNSS) and Individual Nasal Symptoms Scores (NSS)) in Ragweed Allergic Patients Studied in an Environmental Exposure Chamber (EEC) Model. J Allergy Clin Immunol. 2009; 123(2): S130.
3. Fabbri NZ, Abib-Jr E, de Lima Zollner R. Azelastine and Budesonide (nasal sprays): Effect of combination therapy monitored by acoustic rhinometry and clinical symptom score in the treatment of allergic rhinitis. Allergy & Rhinology. 2014; 5(2): 78-86.
4. Fabbri NZ, Zollner RL, Abib E. Azelastine hydrochloride and budesonide (nasal sprays): effectiveness combination therapy monitored by acoustic rhinometry and clinical symptom score in the treatment of allergic rhinitis. Clin Transl Allergy. 2013; 3(Suppl 2):40.
5. Kimura M, Morita Y, Fukushi K. Senju Pharmaceutical Co., Ltd. An aqueous nasal suspension comprising cyclodextrin. European Patent EP 0709099 A2. 1996 May 1.
6. http://www.drugbank.ca/drugs/DB00972.Accessed on: 10 Nov 2015.

7. http://www.drugbank.ca/drugs/DB01222. Accessed on: 10 Nov 2015.
8. www.chemACX.com (Cambridge database). Accessed on: 10 Nov 2015
9. Pipkin J, Zimmerer R, Thompson D, Mosher G. Inhalant formulation containing sulfoalkyl ether cyclodextrin and corticosteroid**.** United States Patent US20070020299 A1. 2007 Jan 25.
10. Gouda AA, Sheikh RE, Saied HE. Extractive spectrophotometric determination of Azelastine Hydrochloride in pure form and pharmaceutical formulations. Canadian Chemical Transactions 2015; 3(1): 29-41.
11. Gouda MM, Shabaraya RA, Shantakumar SM, Shyale SS, Putta R. Development and validation of selective UV spectrophotometric analytical method for budesonide pure sample. J App Pharm Sci. 2011; 1(7): 158-61.
12. Prasad AVSS. Simultaneous spectrophotometric determination of formoterol fumarate and budesonide in their combine dosage form. Indian J Chem Technol. 2006; 13: 81-83.
13. Salama NN, Abdel-Razeq SA, Abdel-Atty S, El-Kosy N. Spectrophotometric determination and thermodynamicstudies of the charge transfer complexes of Azelastine-HCl. Bulletin of Faculty of Pharmacy. 2011; 49: 13-8.
14. Thangabalan B, Palanirajan V. RP-HPLC determination of Azelastine in pure and in ophthalmic formulation. Int J Pharm Sci Rev Res. 2012; 17(2) nº 11: 62-64.
15. Kapadnis RK, Baviskar SR, Mahajan SK. Assay Method Development and validation for simultaneous estimation of Mometasone Furoate and Azelastine Hydrochloride by RP-RPHPLC. Asian J Biochem Pharm Res. 2014; 4(4): 31-39.
16. Dave HN, Makwana AG, Suhagia BN. Validated reversed phase high performance liquid chromatographic method for determination of three novel steroids in bulk and pressurized metered – dose commercial preparations using a common mobile phase. Int J App Sci Engg. 2013; 11(2): 125-35.
17. A. Wikby, L. Nilsson and G. Hallsas. Separation of epimers of budesonide and related corticosteroids by reversed bonded-phase liquid chromatography Journal of Chromatography 1978; 157:51-64.
18. Varshosaz J, Emami J, Tavakoli N, Minaiyan M, Rahmani N, Ahmadi F, Dorkoosh F. Development and validation of a rapid RP-HPLC method for simultaneous analysis of budesonide and its novel synthesized hemiesters in colon specific formulations. Res Pharm Sci. 2011; 6(2): 107-16.

19. Dadhich S, Sharma S, Bhandari A, Agarwal A, Tiwari SK, Nagariya K. Method development and validation for simultaneous estimation of Levosalbutamol Sulphate and Budesonide in bulk and pharmaceutical dosage form by RP-HPLC. Am J PharmTech Res. 2013; 3(2): 939-47.
20. Hou S, Hindle M, Byron PR. Chromatographic and mass spectral characterization of budesonide and a series of structurally related corticosteroids using LC-MS. J Pharm Biomed Anal. 2005; 39: 196-205.
21. Abdel-Razeq SA, Salama NN, Abdel-Atty S, El-Kosy N. Thermoanalytical study and purity determination of Azelastine Hydrochloride and Emedastine Difumarate. Pharmaceut Anal Acta. 2012; 3(8).
22. ICH guidelines, validation of analytical procedure: Methodology Q2B. I.C.H. Harmonized Tripartite Guidelines. Geneva; 1996.

SIMULTANEOUS ESTIMATION OF AZELASTINE HYDROCHLORIDE AND BUDESONIDE IN HUMAN PLASMA BY LC-MS/MS METHOD USING SOLID PHASE EXTRACTION TECHNIQUE

4.1. LITERATURE REVIEW

Various analytical methods have been reported in the literature for the quantitation of AZ and BD either individually or in various combinations with other drugs. The literature for the analytical methods of AZ and BD for their estimation in bulk drug and formulations, has been quoted in Section 3.4. The bioanalytical methods such as determination of AZ by membrane selective electrodes (1), RP-HPLC method for estimation of AZ in human plasma (2), estimation of AZ by electrokinetic capillary chromatography and LC-MS spectrometry (3), LC-MS method for AZ (4-6) and BD (7-15), estimation of BD by gas chromatography (16) have been reported in the literature. There is no bioanalytical method has been reported till date, for the simultaneous estimation of AZ and BD.

4.2. EXPERIMENTAL

4.2.1. Instrumentation

The LC-MS/MS experiment was performed on 3200 QTRAP LC-MS/MS instrument (AB Sciex instruments) equipped with binary pump LC system, Ekspert 100-XL Autosampler, Ekspert 100 Pump, Ekspert 100 Column Oven and Quadrapole mass analyser. The data acquisition was performed through Analyst software (version 1.6.2). The Solid Phase extraction (SPE) was performed on Ezypress^{TM} HT 48 (Orochem Laboratories) SPE assembly attached with nitrogen gas flow. Separation and quantitation were made on an Oyster C8 column (5µ × 4.6 mm i.d.× 250 mm).

4.2.2. Materials and reagents

The API of AZ was procured from Sigma Aldrich and BD was gifted by Avik Pharmaceuticals Ltd., Vapi Gujarat. HPLC grade (Spectrochem) MeOH and ACN were used for HPLC. LC grade Ammoniun formate (LobaChem) and HPLC grade Formic acid (Spectrochem) were used for preparation of buffer. The buffer was prepared in MilliQ water. Drug free human plasma was procured from Suraktam

Blood Bank, Vadodara. Oasis® HLB cartridge (Waters) and Orochem Agility DVB SCX-HL cartridges were used for performing solid phase extraction.

4.2.3. Experimental Conditions

4.2.3.1. Chromatographic conditions

Ammonium formate buffer (0.01 M) was prepared by dissolving 0.63 g of anhydrous ammonium formate in 1 L of MilliQ water. The pH was adjusted to 3.0 using formic acid. The elution was carried out with mobile phase composed of the mixture of ACN and 0.01M ammonium formate buffer (pH 3) in the ratio of 60:40. All determinations were performed at 30 ºC temperature. The flow rate was 1 mL/min. The injection volume was 20 µL. The elution was performed on the Oyster C8 column (5 µ × 4.6 mm × 250 mm i.d.).

4.2.3.2. Mass Parameters details

The details of instrumental parameters for the LC-MS method and parameters for the MRM method developed for AZ and BD have been given in Table 4.1 and 4.2 respectively.

Table 4.1 Instrumental Parameters

Source/gas parameters	Values
Collision gas (CAD)	5
Curtain gas (CUR)	35
Temperature (TEM)	650 ºC
Ion Source	Turbo Spray
Ion Source Gas 1	55
Ion Source Gas 2	50
Ion Spray voltage	5500
Resolution (Q1/Q3)	Unit/Unit
Tray Temperature	30 ºC
Column oven temperature	30 ºC

Table 4.2 Compound Parameters for LCMS

Parameters	AZ	BD	ISTD
Declustering potential (DP)	56	31	91
Entrance potential (EP)	5.5	7	12
Collision Cell Entrance Potential (CEP)	26	18	14
Collision energy (CE)	67	35	43
Collision cell exit potential (CXP)	4	4	4
MRM file parameters	**Scan type**		
Parent (Da)	382	431	295
Daughter (Da)	112	147	213

4.2.4. Preparation of Solutions

4.2.4.1. Preparation of Drug Stock Solutions

AZ stock solution (S-I) (0.856 mg/mL): Accurately weighed 21.4 mg AZ was transferred in 25 mL volumetric flask, dissolved and then diluted with MeOH up to the mark.

AZ working solution (S-II) (0.0856 mg/mL): The S-II was prepared by transferring 2.5 mL from of AZ S-I stock solution to 25 mL volumetric flask and diluted to the mark with mixture of MeOH and water (50:50).

BD stock solution (S-I) (0.2 mg/mL): Accurately weighed 5 mg BD was transferred in 25 mL volumetric flask, dissolved and then diluted with MeOH up to the mark.

BD working solution (S-II) (0.02 mg/mL): The S-II was prepared by transferring 2.5 mL from of BD S-I stock solution to 25 mL volumetric flask and diluted to the mark with mixture of MeOH and water (50:50).

ISTD stock solution (S-I) (1.6 mg/mL): Accurately weighed 40 mg Diclofenac potassium (DC) was transferred in 25 mL volumetric flask, dissolved and then diluted with MeOH up to the mark.

ISTD working solution (S-II) (0.8 mg/mL): The S-II was prepared by transferring 12.5 mL ISTD S-I stock solution to 25 mL volumetric flask and then diluted with mixture of MeOH and water (50:50).

4.2.4.2. Preparation of calibration curve spiking standards

The calibration curve spiking standard (CCSS) solutions were prepared by taking appropriate aliquots from the working stock solutions (S-II) of each of AZ and BD and making up the final volume upto 5 mL with water. The preparation scheme has been given in Table 4.3.

4.2.4.3. Preparation of Spiked Plasma Sample Solutions

The plasma sample solutions (i.e. the final mixture solutions) were prepared by taking appropriate aliquots from the CCSS solutions (the mixture solutions of AZ and BD) and spiking them into the human plasma to make the final volume of 10 mL. The preparation scheme has been given in Table 4.4.

Table 4.3 Calibration Curve Spiking Standards (CCSS)

	AZ			BD		
SAMPLE ID	AZ S-II (ng/mL)	AQ in 5 mL	Final AZ Conc. (ng/mL)	BD S-II (ng/mL)	AQ in 5 mL	Final BD Conc. (ng/mL)
CCSS 1	85600	0.05	856	20000	0.05	200
CCSS 2	85600	0.1	1712	20000	0.1	400
CCSS 3	85600	0.2	3424	20000	0.2	800
CCSS 4	85600	0.4	6848	20000	0.4	1600
CCSS 5	85600	0.6	10272	20000	0.6	2400
CCSS 6	85600	0.8	13696	20000	0.8	3200
CCSS 7	85600	1.0	17120	20000	1.0	4000

AQ=Aliquot, SS=standard solution, ID= identity name for sample

Table 4.4 Spiked Plasma Samples

CCSS SOLN	Aliquot (mL)		Plasma Volume (mL)	Final concentration in 10 mL plasma sample solution			MIXTURE SOLN (ID)
	CCSS	ISTD*		AZ (ng/mL)	BD (ng/mL)	ISTD (ng/mL)	
CCSS 1	0.25	0.25	9.5	21.4	5	20000	MIX1
CCSS 2	0.25	0.25	9.5	42.8	10	20000	MIX2
CCSS 3	0.25	0.25	9.5	85.6	20	20000	MIX3
CCSS 4	0.25	0.25	9.5	171.2	40	20000	MIX4
CCSS 5	0.25	0.25	9.5	256.8	60	20000	MIX5
CCSS 6	0.25	0.25	9.5	342.4	80	20000	MIX6
CCSS 7	0.25	0.25	9.5	428	100	20000	MIX7

SOLN=solution, *Aliquot of ISTD was taken from ISTD S-II solution (800000 ng/mL)

4.2.4.4. Preparation of Spiked QC Samples

The QC samples were prepared by spiking the respective QC spiking solutions in screened human plasma as described in the Table 4.5.

Table 4.5 Spiked QC Samples

CCSS SOLN	Aliquot (mL)		Plasma Volume (mL)	Final concentration in 10ml plasma sample solution			QC SAMPLES (ID)
	CCSS	ISTD		AZ (ng/mL)	BD (ng/mL)	ISTD (ng/mL)	
CCSS 1	0.25	0.25	9.5	21.4	5	20000	LLOQ QC
CCSS 2	0.25	0.25	9.5	42.8	10	20000	LQC
CCSS 4	0.25	0.25	9.5	171.2	40	20000	MQC
CCSS 6	0.25	0.25	9.5	342.4	80	20000	HQC

Aliquot of ISTD was taken from ISTD S-II solution (800000 ng/mL)

4.2.5. ISTD selection

Internal standardisation is commonly used in bioanalytical methods especially with chromatographic procedures. The assumption for the selection of internal standard is similarity between the analyte and internal standard with respect to structure and/or

partition coefficient. Diclofenac was used as the ISTD for the quantification of analytes.

4.2.6. Solid Phase Extraction (SPE) method

The plasma samples/spiked samples were freshly prepared and/ or retrieved from the deep freezer (for stability study) and thawed at room temperature. 1 mL spiked plasma sample solution (as prepared in Table 4.4 and 4.5) was taken and the volume was made upto 2 mL with water. The samples were vortexed for 2 min. The resultant plasma solution was subjected to SPE. The SPE was carried out using the Orochem cartridges. The stepwise procedure for the SPE method has been given below in Table 4.6.

Table 4.6 Steps for Solid Phase Extraction technique

STEP 1	Conditioning the SPE cartridges with 1mL MeOH followed by 1 mL water
STEP 2	Introduction of the plasma sample solution
STEP 3	Complete elution of plasma sample
STEP 4	Extraction phase using mixture of MeOH:ACN:BUFFER (40:30:30)
STEP 5	The extracted solution was dried with Nitrogen fuming
STEP 6	Reconstitution with mobile phase upto 1 mL
STEP 7	Filtration of sample through 0.2 µ membrane filter
STEP 9	Sample analysis through LC-MS.

4.2.7. Method validation

The method validation was performed according to the international guidelines for bioanalytical method validation (17, 18) to evaluate following parameters:

- Linearity of response
- Sensitivity
- Selectivity
- Precision and Accuracy (within-batch and between-batch/inter-day)
- Stabilities including freeze-thaw, bench top, short term and long term stock solution and working solution stability
- Recovery
- Dilution integrity

- Matrix Factor

4.2.7.1. System Suitability

System suitability was performed before start of every new batch. It was performed by injecting six replicates of unextracted MQC samples of AZ, BD and ISTD. The mean standard deviation and %CV for the peak area ratio and for the retention time of analyte and IS were calculated.

4.2.7.2. Auto-Sampler Carryover

For checking auto-sampler carryover, MIX 7 and LLOQ QC were processed; both as per extracted sample preparation and the other set as per the procedure of un-extracted sample preparation (MIX7, LLOQ) and one MIX BL was also processed as per the procedure of extracted sample preparation. These samples along with reconstitution solution (RS) were acquired by auto-sampler in order to check auto-sampler carryover in the following sequence. The sequence of sample analysis for checking carryover has been given in Table 4.7.

Table 4.7 Sequence of samples for auto-sampler carryover

Sr. No.	For un-extracted samples	For extracted samples
1.	RS	Plasma blank*
2.	MIX 7 (UNEX)	MIX 7 (EX) OR ULOQ QC
3.	RS	Plasma blank*
4.	MIX 1 (UNEX)	MIX 1 (EX) OR LLOQ QC

RS=reconstitution solution (mobile phase); UNEX= unextracted
*Plasma blank (without drug and ISTD)

4.2.7.3. Linearity and Accuracy

The linearity of the method was determined by using standard plots associated with 7 point standard curve including at least one LLOQ and one ULOQ. The determinations were performed in triplicates. Each batch consisted of one STD BL, one STD zero (Blank + ISTD) and MIX 1 to MIX 7. Linearity was determined by measuring

regression co-efficient. A straight-line fit was made through the data points by least square regression analysis and a constant proportionality was observed.

Acceptance criteria: Correlation coefficient of the calibration curve should not be less than 0.98. Back-calculated concentration of LLOQ should be within ±20% of nominal concentration. Back-calculated concentration of levels other than LLOQ should be within ±15% of nominal concentration. The percentage of calibration levels meeting acceptance criteria should not be less than 75%. Calibration curve standard(s) failing to meet the above criteria should be excluded from calculation of calibration curve. No two consecutive calibration standards should be failed to meet the above acceptance criteria. No calibration curve standard within acceptance criteria should be excluded from calculation of calibration curve to improve linearity.

4.2.7.4. Accuracy and Precision of Quality Control Samples

Accuracy was evaluated by measuring percentage mean accuracy at each concentration level of calibration curve standard and precision was calculated by measuring %CV at each concentration level of QC.

Acceptance criteria: The % CV of six replicates of each concentration level should be ≤ 15% except for LLOQ, where it should be ≤ 20%. Accuracy of at least 67% of total QC samples should be within ±15% of the respective nominal concentration except for LLOQ QC level, where it should be within ±20% of the respective nominal concentration. Overall accuracy of QC samples at each level should be within ±15% except for LLOQ QC level, where it should be within ±20%.

4.2.7.5. Sensitivity

The sensitivity was evaluated by calculating the precision and accuracy (P &A) of LLOQ QC sample in each of the three acceptable P&A batches individually and in total (between batches).

4.2.7.6. Selectivity

Selectivity was proved by determining two different parameters, namely:

(A) Matrix Effect:

The extracted plasma samples (LQC, HQC) as well as the unextracted samples were analysed in triplicates. %CV was calculated for the samples and the matrix factor was calculated by the formula:-

$$\text{Matrix factor} = \frac{\text{Mean Peak Area in presence of matrix ions}}{\text{Mean Peak Area in absence of matrix ions}}$$

Acceptance criteria: The percentage variability in the matrix factor at each level as measured by the coefficient of variation should be less than 15.00% and overall coefficient of variation for all levels should be less than 15.00%.

(B) Specificity

The specificity of the intended method was established by screening the standard blank plasma (without spiking with drug or internal standard). Six different sources of plasma were analysed. The specificity was evaluated by comparing the responses of interfering peak at the retention time of drugs and ISTD in the standard blank against the response of the respective extracted LLOQ QC sample.

Acceptance criteria: Response of interfering peaks at the retention time of drug should be 20% of the response of respective LLOQ sample. Response of interfering peaks at the retention time of ISTD should be 5% of the response of ISTD of the respective LLOQ sample. At least 80% of the plasma lots should be within the acceptance criteria.

4.2.7.7. Recovery

Recovery for Analytes (AZ and BD)

The percentage mean recoveries were determined by comparing the mean peak area in triplicates for extracted plasma quality control samples at LQC, MQC and HQC against respective mean peak area for triplicates of unextracted quality control samples at LQC, MQC and HQC.

Acceptance criteria: The %CV of replicates at each QC level should be within 15.00%. %CV of % recovery at all levels should be within 15.00%.

Recovery for Internal Standard

The % mean recovery was determined by comparing the mean peak area of internal standard in the extracted plasma quality control samples at MQC against the mean peak area of internal standard in the un-extracted quality control samples.

Acceptance criteria: The %CV of the mean analyte and internal standard recoveries must be ≤ 15% for each quality control samples.

4.2.7.8. Stability

Stability of AZ, BD and ISTD were analysed in different conditions by determining the following parameters:

A. Bench Top Stability

Spiked quality control samples were kept at ambient temperature on working bench for period of 6 hours. Triplicates of each HQC and LQC samples were analysed and the bench top stability was determined

Acceptance criteria: The percentage accuracy for HQC and LQC should be within limits of 85.00 to 115.00%.

B. Freeze Thaw Stability

The freeze thaw stability was performed for three cycles (each cycle of 24 h) and the samples were stored at –70±5°C. Thawing of the samples was done at ambient temperature for about 1 to 2 h. The plasma samples were analysed (in triplicates) after

each cycle and were compared with freshly prepared LQC and HQC samples (analysed in triplicates).

Acceptance criteria: The percentage accuracy for HQC and LQC should be within the acceptance limits of 85.00 to 115.00%.

C. Short Term Stock Solution Stability (STSS)

To assess the stability of stock solution at room temperature, the stock solutions of drugs and ISTD (stability samples) were kept at room temperature on the working bench for a period of about 6 h. After 6 h fresh stock solution of AZ, BD and ISTD were prepared at ULOQ level concentration. Stability and comparison samples were injected for analysis. Results of stability samples were compared with that of freshly prepared comparison samples.

Acceptance criteria: percentage change should be within ±5% for fresh samples when compared with stability samples.

D. Long Term Stock Solution Stability (LTSS)

To assess stock solution stability below 2-8 ºC (refrigerator temperature) for long term, the stock solutions of both the drugs and ISTD were kept in the refrigerator for a period of 12 hours. After 12 hours the ULOQ concentration sample solutions were prepared and the results were compared with the ULOQ samples prepared by dilution of freshly prepared stock solution. The determinations were performed in triplicates and the stability was determined.

Acceptance criteria: percentage change should be within ±5% for fresh samples when compared with stability samples.

E. Dry extract (DE) stability

The DE stability is important whenever the sample processing involves evaporation step. The stability duration was selected on the basis of the characteristic of the analyte and anticipated duration of the difference between the two chromatographic batch runs. The DE stability was performed by using previously processed and dried stability samples. The previously processed (extracted) HQC and LQC samples were dried and kept at -70±5°C for 24 hrs. These samples were analysed thereafter by reconstituting the solution. The analysis was done in triplicate for HQC and LQC

samples and the results were compared with that of freshly prepared LQC and HQC samples (analysed in triplicate).

4.2.7.9. Dilution Integrity (DI)

The dilution integrity was evaluated at 1:2 and 1:4 times dilution. The DI samples were analysed in triplicates each of DQC (1/2) and DQC (1/4) samples. The DQC samples were compared with freshly spiked CC standards. The concentration of the DQC samples was calculated against the comparison samples (CC). The DI was evaluated by ensuring the precision and % mean accuracy of DQC samples as per acceptance criteria.

Acceptance criteria: The precision and accuracy of the dilution integrity QCs should be ≤15% and within ± 15% of the nominal concentrations resp.

4.3. RESULT AND DISCUSSION

4.3.1. Optimisation of extraction method

For the bioanalytical method development, different methods were tried for the extraction of drug from the plasma, such as: protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE). In initial stage of method development, the experiments were performed for the selection of proper extraction procedure. The protein precipitation method was carried out with MeOH, ACN and acetone. The liquid liquid extraction method was carried out with methyl tert butyl ether, ethyl acetate and diethyl ether. The solid phase extraction method was carried out with MeOH, ACN, mixture of ACN and MeOH and mixture of MeOH, ACN and 10 mM ammonium acetate buffer (pH 3). Various types of cartridges viz. Orochem and Oasis cartridge were tried for extraction and the best extraction efficiency was obtained on the Orochem cartridge. Hence Orochem cartridge was selected. The detail of the recoveries obtained with different extracting solvents and methods is given in Table 4.8.

Table 4.8 Recoveries by different extraction techniques

Method	Extracting Solvent	% Recovery (%)	
		AZ	BD
Protein Precipitation	MeOH	53-55	80-82
	ACN	65-67	60-62
	Acetone	45-47	50-52
Liquid- Liquid Extraction	MTBE	58-60	38-40
	Ethyl acetate	28-30	45-47
	Diethyl ether	30-32	46-48
Solid Phase Extraction	MeOH	55-57	80-82
	ACN	71-73	57-59
	Mixture of MeOH: ACN (50:50)	73-75	63-65
	*Mixture of MeOH, ACN and 10 mM ammonium acetate buffer (pH 3) [40:30:30]	82-84	88-90

*Final selected extraction method

4.3.2. Internal Standard

Various internal standards such as Telmisartan, Tadalafil, Simvastatin, Nifedipine, Nepafenac, Diclofenac, etc. were tried in accordance with the similarity of pKa and partition coefficient of AZ and BD. Among these the best suitability was obtained with Diclofenac in terms of resolution, peak symmetry and elution. Hence among all the moieties screened for ISTD, Diclofenac was selected as ISTD due to stable response and symmetrical peak under the experimental condition with appropriate retention time at 8.7 min. It gave suitable resolution with both the drugs and matrix effect was not observed.

4.3.3. MS scan files

The tuning of APIs and ISTD was done as per the optimised parameters shown in Table 4.1. The scan of the mass spectra of parent and daughter ion is shown in Figure 4.1-4.6.

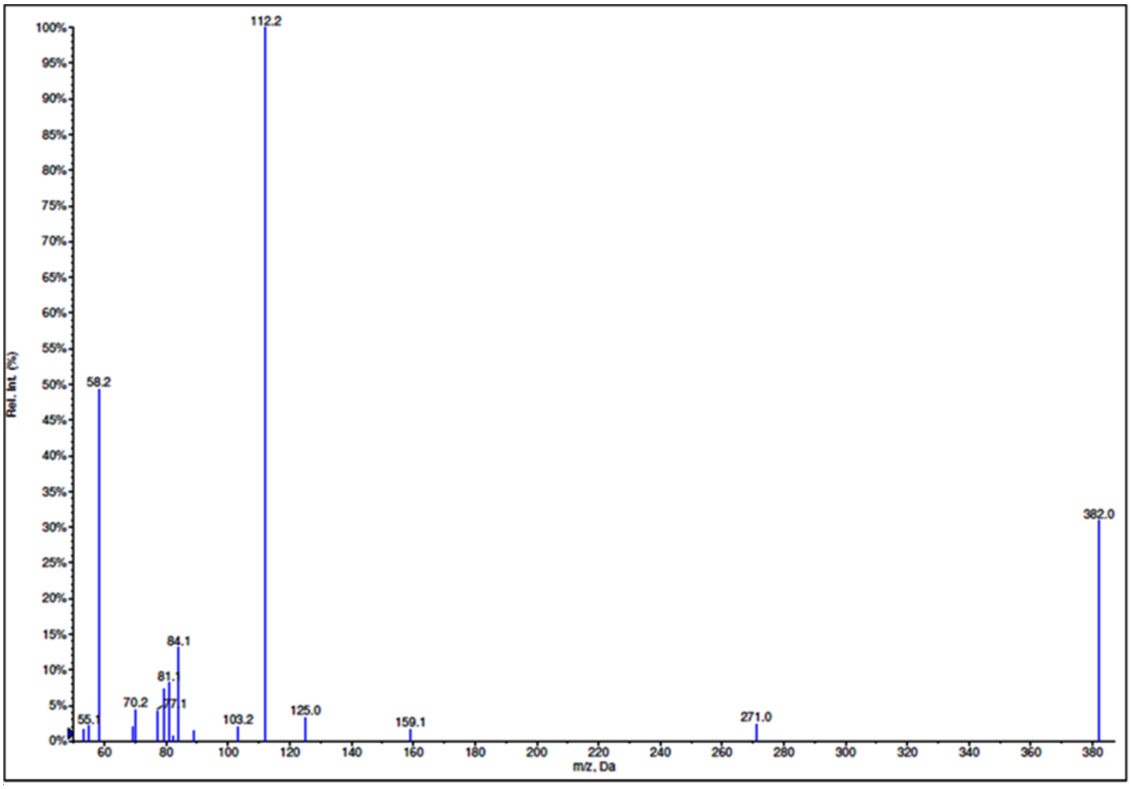


Figure 4.1 Parent Scan: AZ

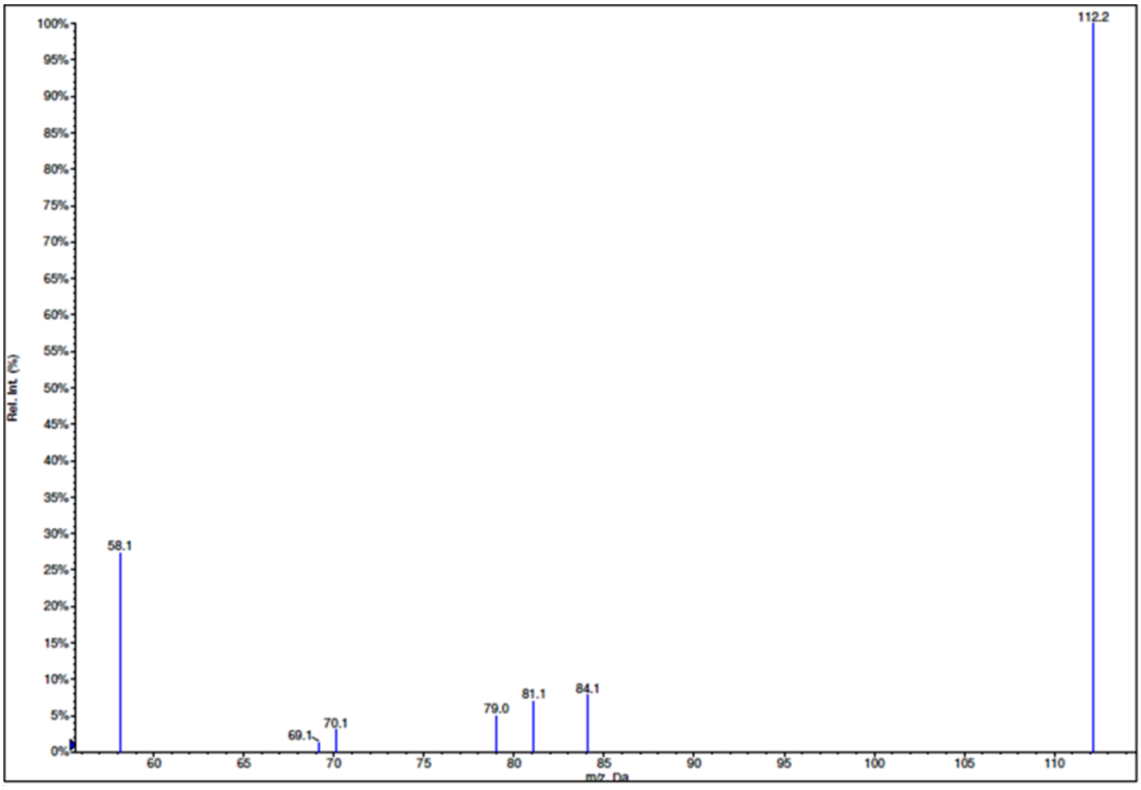


Figure 4.2 Daughter Scan: AZ

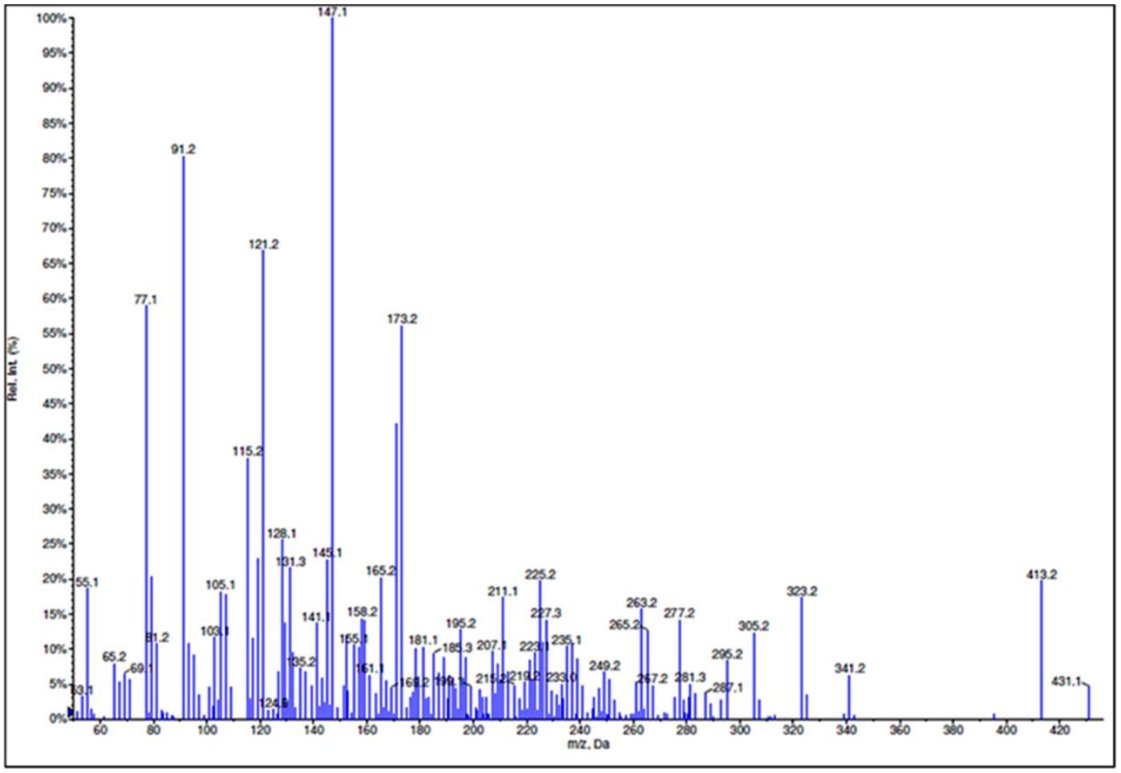


Figure 4.3 Parent Scan: BD

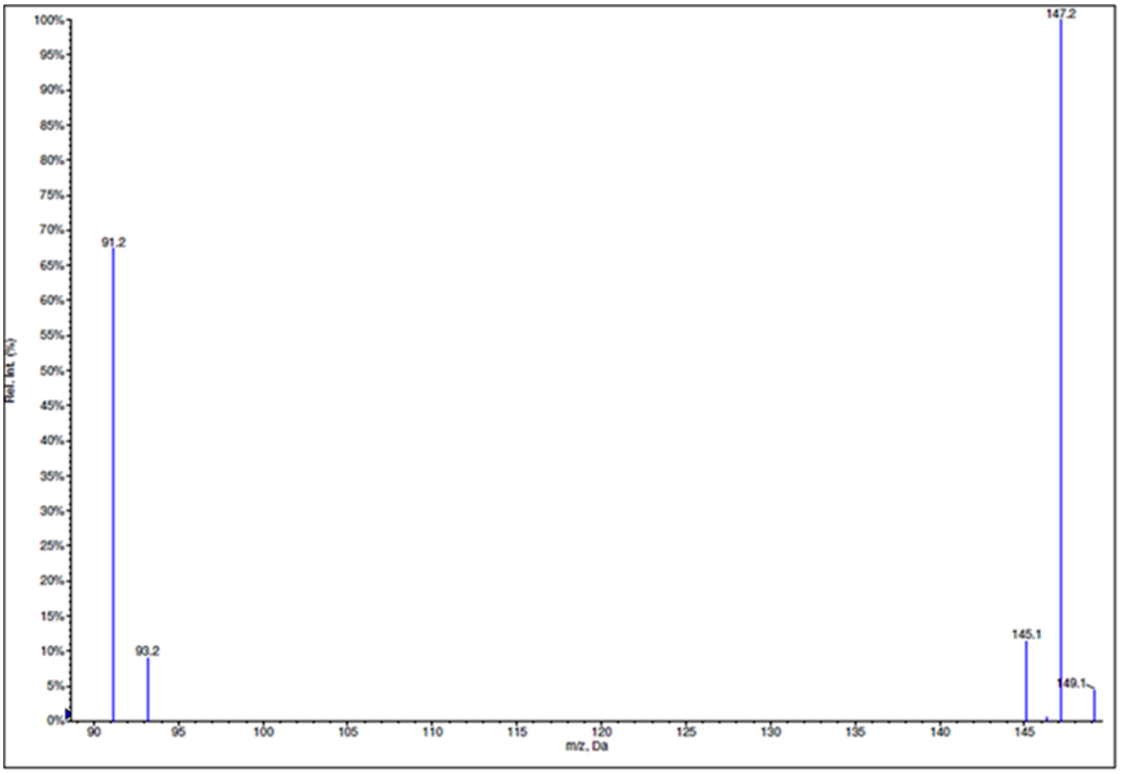


Figure 4.4 Daughter Scan: BD

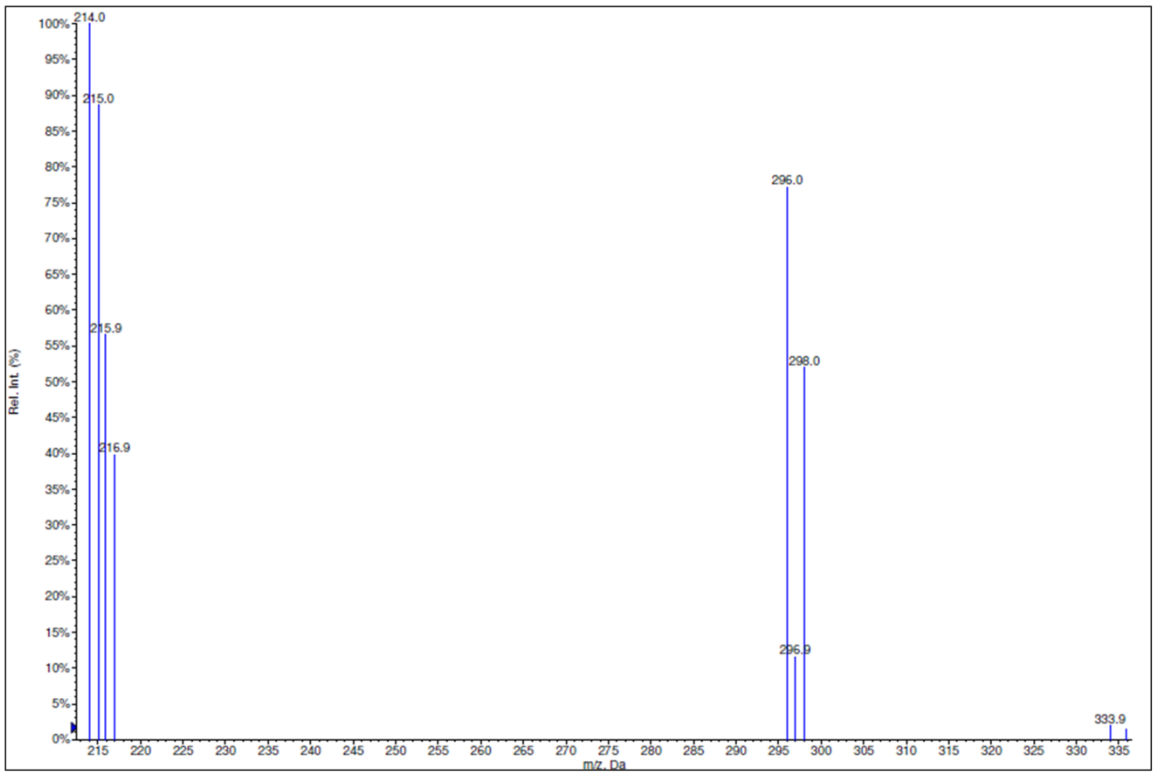


Figure 4.5 Parent Scan: ISTD

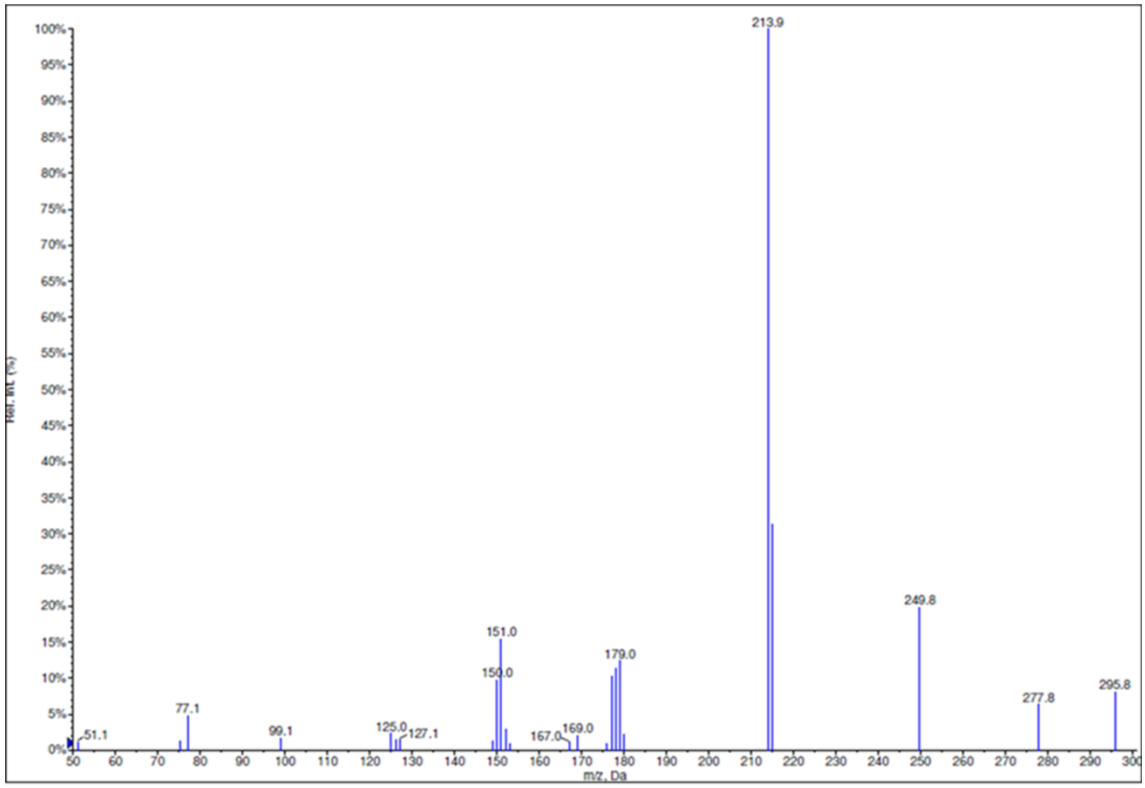


Figure 4.6 Daughter Scan: ISTD

4.3.4. Method Validation

Results of method validation during optimization of various parameters are discussed below.

4.3.4.1 System Suitability

The %CV of system suitability was observed in the range of 0.12-0.24%, 0.11-0.15% and 0.09-0.17% for the retention times of AZ, BD and ISTD respectively. The area ratio was found to be in the range of 0.26-0.47 and 0.37-0.47 for AZ and BD respectively. The %CV range was within 5.00% range as per the acceptance criteria. The data of one day has been given in Table 4.9 and the combined data for all three days has been given in Table 4.10.

Table 4.9 System suitability data for DAY 1

Sample name	AZ Area Ratio	AZ RT	BD Area Ratio	BD RT	ISTD RT
MQC-1	0.897	4.13	0.0145	7.11	8.79
MQC-2	0.920	4.12	0.0140	7.11	8.78
MQC-3	0.858	4.13	0.0143	7.13	8.79
MQC-4	0.888	4.12	0.0137	7.10	8.79
MQC-5	0.904	4.12	0.0144	7.13	8.78
MQC-6	0.924	4.13	0.0145	7.12	8.76
Mean	0.89	4.13	0.01	7.12	8.78
STDV	0.02	0.01	0.0003	0.01	0.012
% CV	2.67	0.13	2.23	0.17	0.13

Table 4.10 System suitability data for three days

DAY	%CV				
	AZ RT	BD RT	ISTD RT	AZ Area Ratio	BD Area Ratio
Day 1	0.13	0.17	0.13	2.67	2.23
Day 2	0.12	0.13	0.09	3.38	2.45
Day 3	0.19	0.15	0.17	3.26	3.37

4.3.4.2. Auto-Sampler Carryover

The auto-sampler carryover was not found, as the drugs as well as ISTD were not carried over in the blank injection given subsequently after sample injection. The results are shown in Table 4.11. Figure 4.7 and 4.8 shows the chromatogram for standard blank and blank plasma sample after ULOQ injection.

Table 4.11 Auto sampler carryover results for Extracted samples

Sample	Peak Area AZ		Peak Area BD		Peak Area ISTD		% Carry-over
	(UNEX)	(EX)	(UNEX)	(EX)	(UNEX)	(EX)	
Blank	0.00	0.00	0.00	0.00	0.00	0.00	NA
MIX 7	3253765.45	2769476.25	49665.53	44739.64	2058376.04	1343735.16	
Blank	0.00	0.00	0.00	0.00	0.00	0.00	NIL
MIX 1	404264.53	325474.26	4592.52	4025.23	2048631.43	1339573.82	

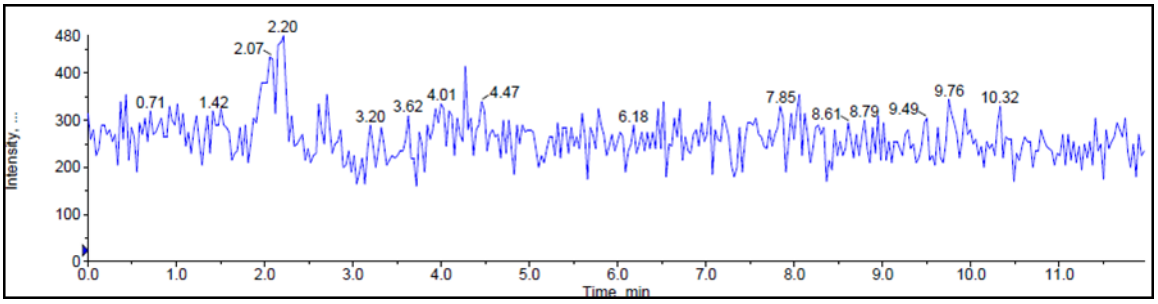


Figure 4.7 Chromatogram for standard blank

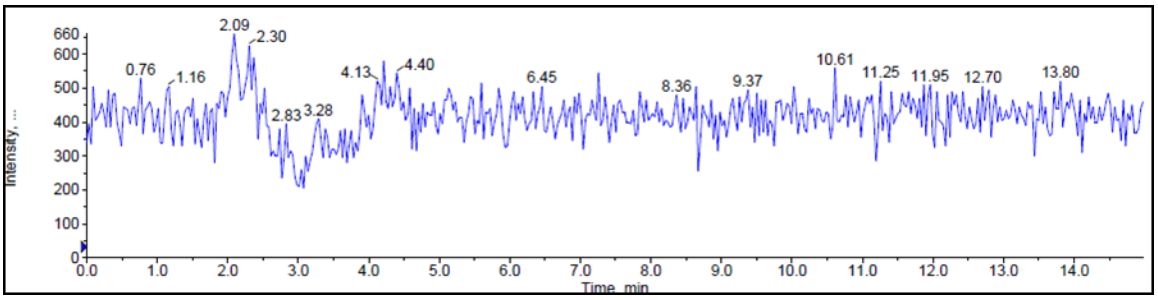


Figure 4.8 Chromatogram for blank plasma

4.3.4.3. Linearity, Accuracy and Precision of Calibration Curve Standards

The linearity of the method was determined by regression analysis of standard plots associated with seven standard samples (extracted from plasma). The calibration curves analysed during the course of validation were linear for the standards ranging from 21.4 to 428 ng/mL for AZ and 5 to 100 ng/mL for BD. The correlation coefficient (r^2) was observed to be 0.9989 and 0.9983 for AZ and BD respectively. The mean accuracy observed for the mixture solutions (MIX 1-7) ranged from 95.05 to 105.62% for AZ and 94.67 to 104.04% for BD, which was within the acceptance limits of 85.00 to 115.00% for all mixture solutions. The LLOQ standard accuracy was found to be 102.5% for AZ and 94.67% for BD which was also within the acceptance limit of 80.00 to 120.00%. The precision observed for the mixture solutions ranged from 0.62 to 5.1% for AZ and 0.4 to 5.5% for BD, which was within the acceptance limit of 15.00% and for the LLOQ standard it was 5.1% for AZ and 5.5% for BD which was also within the accepted criteria of 20.00%. The results of linearity, accuracy and precision have been shown in Table 4.12. The calibration curves of AZ and BD for standard mixture (unextracted) has been shown in Figure 4.9. The calibration curves for AZ and BD in plasma and drug: ISTD ratio are shown in Figure 4.10 and 4.11 for AZ and BD respectively. The chromatograms for mixtures have been shown in section 4.3.4.10.

Table 4.12 Back Calculated Concentrations for Calibration Curve Standards

AZ							
MIX ID	MIX1	MIX2	MIX3	MIX4	MIX5	MIX6	MIX7
Nominal conc.	21.4	42.8	85.6	171.2	256.8	342.4	428
1	23.22	41.79	90.96	164.28	250.26	347.41	421.99
2	21.20	40.95	89.84	165.96	254.45	351.95	427.50
3	21.38	39.31	90.43	171.90	251.38	354.21	424.31
SD	1.12	1.26	0.56	4.00	2.17	3.46	2.77
Avg	21.93	40.68	90.41	167.38	252.03	351.19	424.60
%CV	5.10	3.10	0.62	2.39	0.86	0.99	0.65
%Mean Accuracy	102.50	95.05	105.62	97.77	98.14	102.57	99.21
BD							
MIX ID	MIX1	MIX2	MIX3	MIX4	MIX5	MIX6	MIX7
Nominal conc.	5	10	20	40	60	80	100
1	4.92	10.04	21.00	39.97	58.47	82.84	98.58
2	4.44	9.81	20.66	39.61	58.29	81.16	101.38
3	4.84	10.04	20.77	39.29	58.01	84.48	96.41
SD	0.26	0.13	0.17	0.34	0.23	1.66	2.49
Avg	4.73	9.96	20.81	39.62	58.26	82.83	98.79
%CV	5.51	1.29	0.83	0.86	0.40	2.01	2.52
%Mean Accuracy	94.67	99.62	104.04	99.05	97.10	103.53	98.79

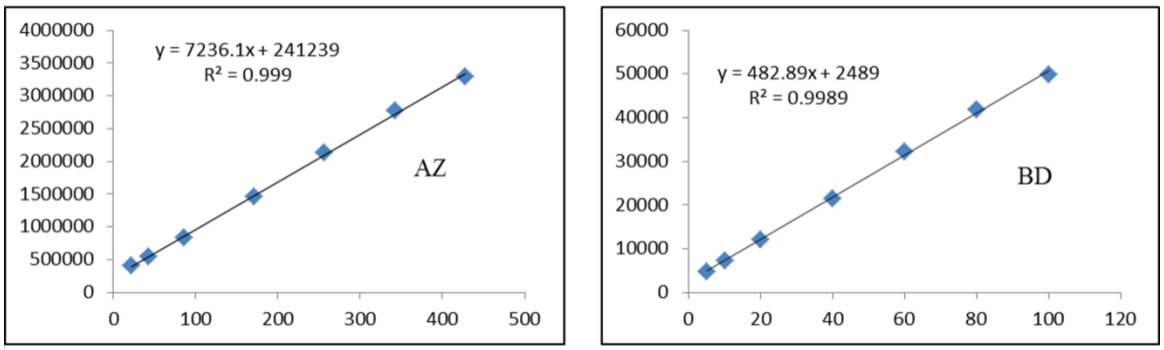


Figure 4.9 Calibration curves for AZ and BD ratio for standard mixture (unextracted) [Peak area vs Conc. (ng/mL)]

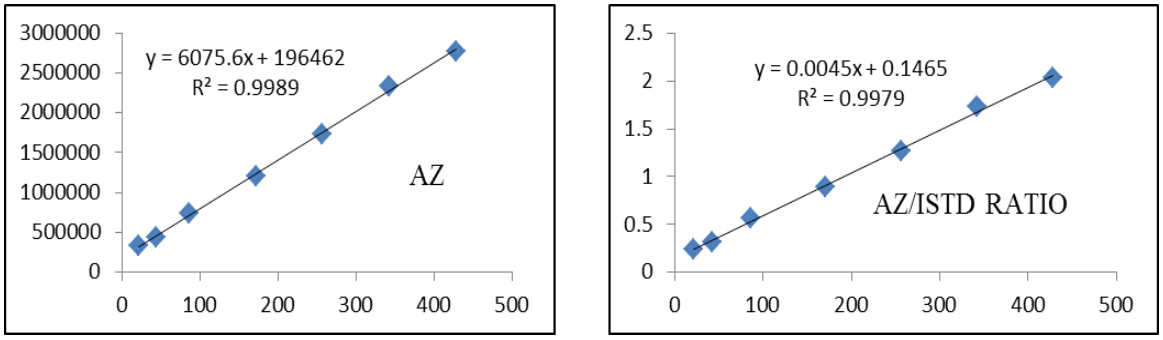


Figure 4.10 Calibration curves for AZ [Peak area vs Conc. (ng/mL)] and AZ: ISTD ratio in human plasma [Peak area ratio AZ/ISTD vs Conc. of AZ (ng/mL)]

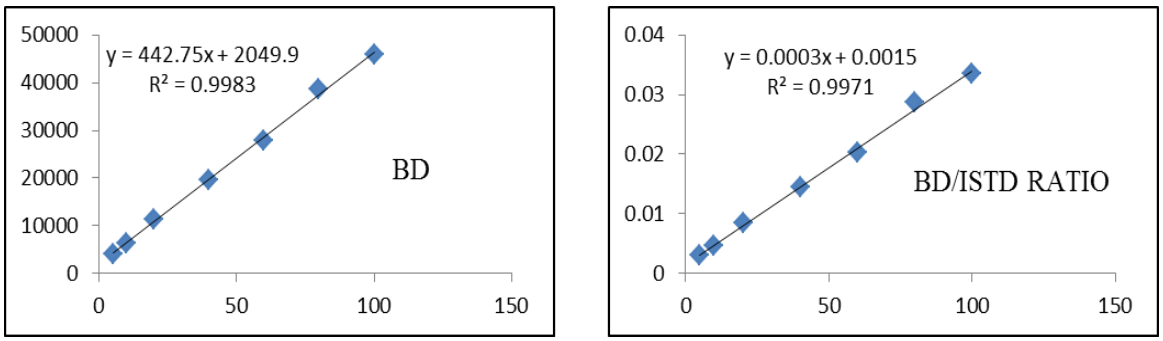


Figure 4.11 Calibration curves for BD [Peak area vs Conc. (ng/mL)]and BD: ISTD ratio in human plasma [Peak area ratio BD/ISTD vs Conc. of BD (ng/mL)]

4.3.4.4. Accuracy and Precision of Quality Control Samples

The results of accuracy and precision for AZ and BD are given in Table 4.13 and 4.14 respectively.

Within Batch Accuracy

The within batch accuracy for the low, middle and high quality control samples ranged from 99.27 to 99.85% for AZ and 98.06 to 98.83% for BD which was within the acceptance limits of 85.00 to 115.00%. The within batch accuracy for the LLOQ quality control sample was 98.27% for AZ and 101.57% for BD which was within the acceptance limits of 80.00 to 120.00%. The overall accuracy was found to be 99.34% and 99.19% for AZ and BD resp. which was within ±15% limits.

Between Batch Accuracy

The between batch accuracy for the low, middle and high quality control samples ranged from 98.56 to 100.66% for AZ and 97.53 to 98.76% for BD which was within the acceptance limits of 85.00 to 115.00%. The between batch accuracy for the LLOQ quality control samples was 98.17% for AZ and 100.96% for BD which was within the acceptance limits of 80.00 to 120.00%. The overall accuracy was found to be 99.21% and 98.95% for AZ and BD resp. which was within ±15% limits.

Within Batch Precision

The within batch precision for the low, middle and high quality control samples ranged from 0.37 to 0.56% for AZ and 1.17 to 3.68% for BD which was within the acceptance limit of 15.00%. The within batch precision for the LLOQ quality control sample was 1.99% for AZ and 0.43% for BD which was within the acceptance limit of 20.00%.

Between Batch Precision

The between batch precision for the low, middle and high quality control samples ranged from 0.5 to 0.72% for AZ and 1.42 to 2.4% for BD which was within the acceptance limit of 15.00%. The between batch precision for all the LLOQ quality control samples was from 2.5 for AZ and 0.59% for BD which was within the acceptance limit of 20.00%.

Table 4.13 Results of Accuracy and Precision of Quality Control Samples for AZ

	HQC	MQC	LQC	LLOQ QC
Within batch precision and accuracy of AZ				
1	2314478.56	1209385.68	445684.74	321948.57
2	2329437.65	1213475.56	440837.68	318844.91
3	2329483.54	1199484.56	442347.29	331245.03
Mean	2324466.58	1207448.60	442956.57	324012.84
STDV	8649.91	7193.83	2480.31	6452.65
%CV	0.37	0.60	0.56	1.99
%Mean Accuracy	99.76	99.51	99.85	98.27

Between batch precision and accuracy of AZ				
1	2324466.58	1207448.60	442956.57	324012.84
2	2332743.92	1197749.35	448424.99	315451.33
3	2310003.33	1215154.49	448252.29	331659.88
Mean	2296670.00	1206784.14	446544.62	323708.02
STDV	11509.67	8721.57	3108.54	8108.57
%CV	0.50	0.72	0.70	2.50
%Mean Accuracy	98.56	99.46	100.66	98.17

Table 4.14 Results of Accuracy and Precision of Quality Control Samples for BD

Within batch precision and accuracy of BD				
	HQC	MQC	LQC	LLOQ QC
1	37729.82	19783.34	6384.73	4230.20
2	37693.58	18451.83	6492.17	4194.56
3	38482.47	19538.94	6277.75	4206.68
Mean	37968.62	19258.04	6384.89	4210.48
STDV	445.37	708.81	107.21	18.12
%CV	1.17	3.68	1.68	0.43
%Mean Accuracy	98.06	98.30	98.83	101.57
Between batch precision and accuracy of BD				
1	37968.62	19258.04	6384.89	4210.48
2	37154.31	19798.43	6267.57	4161.22
3	38169.24	18876.91	6489.27	4184.13
Mean	37764.06	19311.12	6380.58	4185.28
STDV	537.50	463.05	110.91	24.65
%CV	1.42	2.40	1.74	0.59
%Mean Accuracy	97.53	98.57	98.76	100.96

4.3.4.5. Selectivity

(A) Matrix Effect: The matrix effect for the intended method was assessed by using six sources of human plasma and determining the HQC and LQC samples in absence and presence of matrix. Matrix factor range for QC samples was in the range of 0.81 to 0.85 for AZ, 0.84 to 0.88 for BD and 0.65 to 0.66 for ISTD. The % variance for AZ, BD and ISTD was within the acceptance criteria (±15%). The results of matrix effects have been given in Table 4.15, 4.16 and 4.17.

Table 4.15 Results of matrix effect for AZ

Sr. No.	Response for AZ			
	(in absence of matrix)		(in presence of matrix)	
	HQC	LQC	HQC	LQC
1	2769446.39	552891.69	2327799.92	443622.67
2	2749376.45	556923.23	2337563.45	458263.88
3	2773946.52	573368.34	2347354.16	461493.23
4	2804346.65	563937.54	2378945.73	472237.41
5	2794745.76	572474.76	2369755.27	452574.33
6	2748236.45	548345.67	2357452.91	425645.65
Mean	2773349.70	561323.54	2353145.24	452306.19
SD	22976.45	10345.47	19384.68	16145.64
%CV	0.83	1.84	0.82	3.57
Matrix factor			0.85	0.81

Table 4.16 Results of matrix effect for BD

Sr. No.	Response for BD			
	(in absence of matrix)		(in presence of matrix)	
	HQC	LQC	HQC	LQC
1	41826.09	7281.98	36555.87	6431.07
2	43859.40	7492.54	37196.18	6492.85
3	44748.45	7317.37	36429.74	6627.17
4	43634.51	7483.55	37957.84	6638.70
5	42589.34	7358.23	35877.23	6491.85
6	42685.75	7146.43	34926.47	6259.10
Mean	43223.92	7346.68	36490.55	6490.12
SD	1052.89	130.60	1047.03	139.80
%CV	2.44	1.78	2.87	2.15
Matrix factor			0.84	0.88

Table 4.17 Results of matrix effect for ISTD

Sr. No.	Response for ISTD			
	(in absence of matrix)		(in presence of matrix)	
	HQC	LQC	HQC	LQC
1	2048465.93	2057363.46	1358343.14	1373965.56
2	2068245.25	2073457.34	1348548.65	1353784.64
3	2081474.64	2067342.72	1374674.45	1347335.45
4	2053546.35	2073284.30	1356268.38	1326857.27
5	2045659.34	2084642.56	1337529.57	1336835.56
6	2069478.60	2062274.45	1347378.44	1369363.56
Mean	2061145.02	2069727.47	1353790.44	1351357.01
SD	14081.62	9624.20	12617.57	18267.78
%CV	0.68	0.46	0.93	1.35
Matrix factor			0.66	0.65

(B) Specificity

The specificity of the intended method was established by screening the standard blank plasma (without spiking with drug or internal standard). Six different sources of plasma were analysed and the LLOQ concentrations of AZ, BD and ISTD were determined.The interference of plasma at the retention of the peak of interest *i.e.* peak of AZ, BD and ISTD was examined by analysing the LLOQ QC sample after the blank plasma injection. The results are shown in Table 4.18. From the results it was seen that there was no significant interference observed by matrix ions or plasma on retention time and response of drug and ISTD.

Table 4.18 Results for specificity

Source	Area AZ	% Int	Area BD	% Int	Area ISTD	% Int
1	319463.54	0.02	3927.56	0.02	134366.65	0.01
2	328465.30	0.01	4092.25	0.01	136784.45	0.01
3	319637.45	0.01	4194.85	0.00	135378.33	0.00
4	329663.74	0.00	3978.65	0.00	136348.14	0.00
5	325128.54	0.01	4184.53	0.00	135854.86	0.01
6	328378.95	0.01	4015.34	0.00	134964.43	0.00

Int=Interferance

4.3.4.6. Recovery

The percentage mean recovery for was found to be 83.97%, 88.66 and 66.32% for AZ, BD and ISTD respectively. The %CV was in the range of 0.22-1.19% for AZ and 0.88-3.78% for BD which was within the acceptance criteria of ±15%. The results of recovery have been stated in Table 4.19, 4.20 and 4.21 for AZ, BD and ISTD respectively.

Table 4.19 Recovery results for AZ

Replicate No.	HQC		MQC		LQC	
	(EX)	(UNEX)	(EX)	(UNEX)	(EX)	(UNEX)
1	2324478.56	2772387.45	1239343.00	1469385.68	449684.00	520373.00
2	2319437.65	2817213.12	1229384.00	1453475.56	440837.00	539373.00
3	2329483.54	2788659.17	1248723.22	1479484.56	450347.00	531929.07
%CV	0.22	0.81	1.06	0.66	1.19	1.80
% Mean recovery	83.23		84.44		84.24	
Overall % Mean recovery ± %CV	83.97 ± 0.77					

EX=extracted; UNEX=unextracted

Table 4.20 Recovery results for BD

Replicate No.	HQC		MQC		LQC	
	(EX)	(UNEX)	(EX)	(UNEX)	(EX)	(UNEX)
1	36695.64	42703.34	18393.64	20938.13	6493.65	7334.23
2	39537.30	41103.64	18594.23	21957.20	6395.03	7244.42
3	37734.66	42771.29	17642.50	19734.60	6493.54	7267.28
%CV	3.78	2.24	2.76	5.33	0.88	0.64
% Mean recovery	90.04		87.23		88.72	
Overall % Mean recovery ± %CV	88.66 ± 1.58					

EX=extracted; UNEX=unextracted

Table 4.21 Recovery results for ISTD

Replicate No.	HQC		MQC		LQC	
	(EX)	(UNEX)	(EX)	(UNEX)	(EX)	(UNEX)
1	1348662.65	2048462.46	1386459.46	2094634.65	1385484.01	2038564.24
2	1373707.56	2079453.65	1379703.75	2118464.76	1355835.00	1986456.76
3	1348464.56	2063964.73	1363890.65	2104745.15	1379453.65	2047464.76
%CV	1.07	0.75	0.84	0.57	1.14	1.63
% Mean recovery	65.74		65.37		67.86	
Overall % Mean recovery ± %CV	66.32± 2.02					

EX=extracted; UNEX=unextracted

4.3.4.7. Stability Studies

A. Bench Top Stability:

The % accuracy for HQC and LQC were 100.13 to 102.05% and 96.82 to 97.26% for AZ and 95.79 to 99.80% and 97.73 to 102.58% for BD respectively, which were within the acceptance limits of 85.00 to 115.00%. The results have been given in Table 4.22.

Table 4.22 Bench Top Stability Results

	AZ		**BD**	
Nominal Conc.	(HQC=342.4 ng/mL) (LQC=42.8 ng/mL)		(HQC= 80 ng/mL) (LQC=10 ng/mL)	
Sample ID	Calculated Concentration (ng/mL)	% Accuracy	Calculated Concentration (ng/mL)	% Accuracy
BTHQC1	347.61	101.52	79.83	99.80
BTHQC2	342.83	100.13	76.63	95.79
BTHQC3	349.42	102.05	79.66	99.57
BTLQC1	41.63	97.26	10.26	102.58
BTLQC2	41.44	96.82	9.77	97.73
BTLQC3	41.50	96.97	9.99	99.87

B. Freeze Thaw Stability

Freeze thaw stability of the spiked quality control samples was carried out for three cycles below -70±5ºC. The % accuracy for HQC and LQC was in the range of 98.56 to 99.96% and 93.59 to 96.15% for AZ and 94.56 to 99.09% and 95.2 to 98.65% for BD which were within the acceptance limits of 85.00 to 115.00%. The results have been given in Table 4.23.

Table 4.23 Freeze Thaw Stability Results (Below -70±5ºC)

	AZ		**BD**	
Nominal Conc.	(HQC=342.4 ng/mL) (LQC=42.8 ng/mL)		(HQC= 80 ng/mL) (LQC=10 ng/mL)	
Sample ID	Calculated Concentration (ng/mL)	% Accuracy	Calculated Concentration (ng/mL)	% Accuracy
FTHQC1	342.27	99.96	79.27	99.09
FTHQC2	338.81	98.95	78.38	97.98
FTHQC3	337.48	98.56	75.65	94.56
FTLQC1	41.15	96.15	9.87	98.65
FTLQC2	40.06	93.59	9.80	97.95
FTLQC3	40.60	94.87	9.52	95.20

Average of each cycle for three days (day 1, 2, 3)

C. Short Term Stock Solution Stability (STSS)

The stock solutions of AZ, BD and ISTD were found to be stable for 6 hours. The percentage mean stability was 99.06, 99.91 and 99.13% for AZ, BD and ISTD respectively. The results have been given in Table 4.24.

D. Long Term Stock solution stability (LTSS)

The stock solutions of AZ, BD and ISTD were found to be stable for 12 hours. The percent mean stability was 99.88, 97.31 and 98.82% for AZ, BD and ISTD respectively. The results have been given in Table 4.25.

Table 4.24 Short Term Stock Solution Stability Results

	AZ		BD		ISTD	
Nominal Conc.	428 ng/mL		100 ng/mL		20000 ng/mL	
Sample No.	CS	SS	CS	SS	CS	SS
STSS1	3314485.34	3291948.44	50593.56	49735.75	1969734.45	1949574.56
STSS2	3309485.56	3277286.26	50355.34	49838.34	1979455.12	1969743.34
STSS3	3294596.34	3256583.68	49604.65	50846.35	1975435.34	1953846.44
Mean	3306189.08	3275272.79	50184.52	50140.14	1974874.97	1957721.45
SD	10346.17	17768.15	516.11	613.74	4884.50	10628.11
%CV	0.31	0.54	1.03	1.22	0.25	0.54
%Mean Stability	99.06		99.91		99.13	

CS=comparison samples, SS=stability sample

Table 4.25 Long Term Stock solution stability Results

	AZ		BD		ISTD	
Nominal Conc.	428 ng/mL		100 ng/mL		20000 ng/mL	
Sample No.	CS	SS	CS	SS	CS	SS
LTSS1	3314485.34	3286513.74	50593.56	49863.53	1969734.45	1939575.43
LTSS2	3309485.56	3325025.65	50355.34	47864.32	1979455.12	1950346.56
LTSS3	3294596.34	3294794.56	49604.65	48775.35	1975435.34	1964734.67
Mean	3306189.08	3302111.32	50184.52	48834.40	1974874.97	1951552.22
SD	10346.17	20271.73	516.11	1000.91	4884.50	12622.88
%CV	0.31	0.61	1.03	2.05	0.25	0.65
%Mean Stability	99.88		97.31		98.82	

E. Dry Extract stability

The percentage accuracy of the dry extract stability samples was found in the range of 95.10 to 99.26% for AZ and 95.96 to 99.67% for BD which was within ±5% limit. The results have been given in Table 4.26.

Table 4.26 Dry Extract stability Results

	AZ		**BD**	
Nominal Concentration	(HQC = 342.4 ng/mL) (LQC = 42.8 ng/mL)		(HQC = 80 ng/mL) (LQC = 10 ng/mL)	
Sample ID	Calculated Concentration (ng/mL)	% Accuracy	Calculated Concentration (ng/mL)	% Accuracy
DEHQC1	339.57	99.17	79.73	99.67
DEHQC2	339.86	99.26	78.46	98.07
DEHQC3	339.59	99.18	78.52	98.15
DELQC1	41.11	96.05	9.60	95.96
DELQC2	40.70	95.10	9.62	96.23
DELQC3	41.07	95.95	9.88	98.84

4.3.4.8. Dilution Integrity

The precision of dilution integrity QC (DQC) samples for 1:2 and 1:4 dilution was 2.97% and 1.41% for AZ and 1.88% and 3.04% for BD respectively. The % mean accuracy of DQC samples ranged from 96.09 to 98.44% for AZ and 95.17 to 97.85% for BD for the two dilutions as specified. The results have been given in Table 4.27 and satisfied the acceptance criteria.

Table 4.27 Dilution Integrity of AZ and BD

	AZ		BD	
Nominal conc.	1:2 dilution	1:4 dilution	1:2 dilution	1:4 dilution
(ng/mL)	214	42.8	50	10
	Back calculated conc.(ng/mL)			
DQC1	210.45	41.54	48.72	9.83
DQC2	198.76	42.73	48.13	9.26
DQC3	207.68	42.13	49.93	9.46
Mean	205.63	42.13	48.93	9.52
SD	6.11	0.60	0.92	0.29
%CV	2.97	1.41	1.88	3.04
%mean accuracy	96.09	98.44	97.85	95.17

4.3.4.9. Summary of validation parameters

The results of the bioanalytical method validation have been summarised in Table 4.28.

Table 4.28 Summary of validation parameters

Parameters	AZ	BD
Calibration range (ng/mL)	21.4-428	5-100
LLOQ (ng/mL)	21.4	5
Regression equation (for Drug)	y = 6075.6x +196462	y = 442.75x +2049.9
Correlation coefficient (r^2)	0.9989	0.9981
Regression equation (for ratio of Drug/ISTD)	y = 0.0045x + 0.1465	y = 0.0003x + 0.0015
Correlation coefficient (r^2)	0.9979	0.9971
Recovery	83.97 ± 0.77	88.66 ± 1.58

*STSS		99.06 ± 0.24	99.91 ± 2.25
*LTSS		99.88 ± 0.67	97.31 ± 2.01
*Within Batch	HQC	99.76 ± 0.37	98.06 ± 1.17
	MQC	99.51 ± 0.60	98.3 ±3.68
	LQC	99.85 ± 0.56	98.83 ±1.68
	LLOQ QC	98.27 ± 1.99	101.57 ±0.43
*Between Batch	HQC	98.56 ± 0.5	97.53 ±1.42
	MQC	99.46 ± 0.72	98.57 ± 2.4
	LQC	100.66 ± 0.7	98.76 ± 1.74
	LLOQ QC	98.17 ± 2.5	100.96 ± 0.59
#Matrix Effect	HQC	0.84	0.87
	MQC	0.82	0.9
	LQC	0.8	0.88
*Bench Top Stability	HQC	101.23 ± 0.98	98.39 ± 2.28
	LQC	97.02 ± 0.23	100.06 ± 2.45
*Freeze Thaw Stability	HQC	99.16 ± 0.73	97.21 ± 2.43
	LQC	94.87 ± 1.34	97.27 ± 1.9
*Dilution Integrity	HQC	96.09 ± 2.97	97.85 ± 1.88
	LQC	98.44 ± 1.41	95.17 ± 3.04
*Dry Extract Stabiliy	HQC	99.2 ± 0.058	98.63 ± 0.91
	LQC	95.7 ± 0.55	97.01 ± 1.61

*Average of three determinations±%CV
#Average of three determinations

4.3.4.10. Chromatograms

The figures of the MRM mode peaks for plasma samples of MIX1-7 have been given in the Figures 4.12-4.18 for AZ and BD and Figure 4.19-4.25 for ISTD.

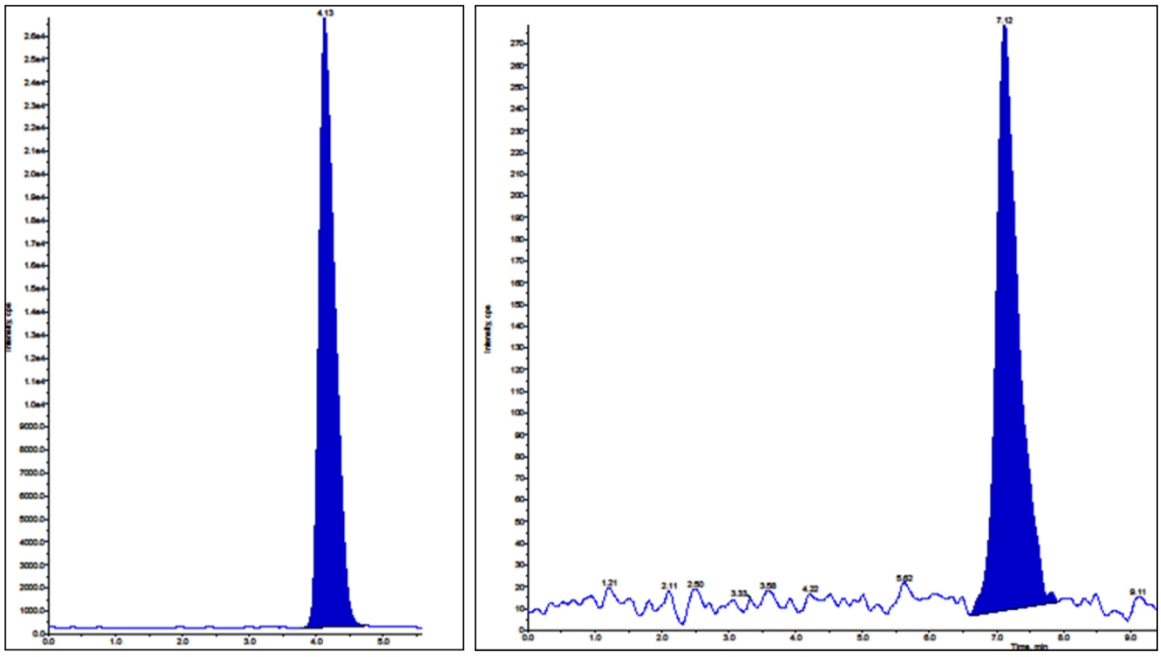


Figure 4.12 Chromatogram for AZ and BD [MIX 1]

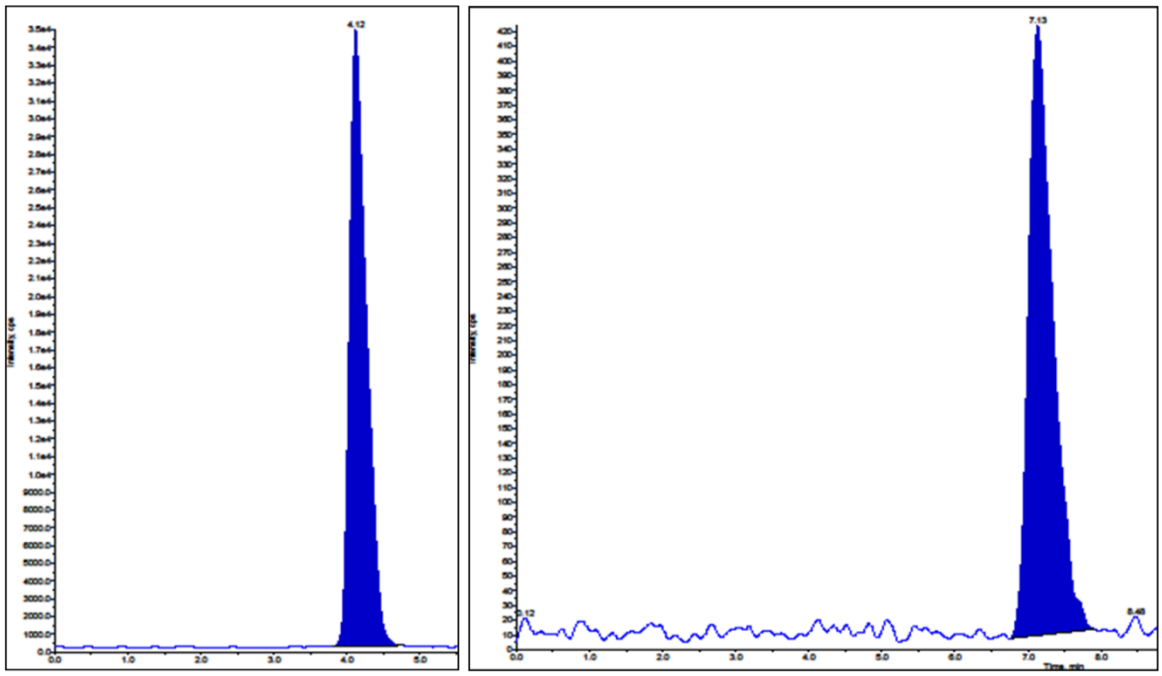


Figure 4.13 Chromatogram for AZ and BD [MIX 2]

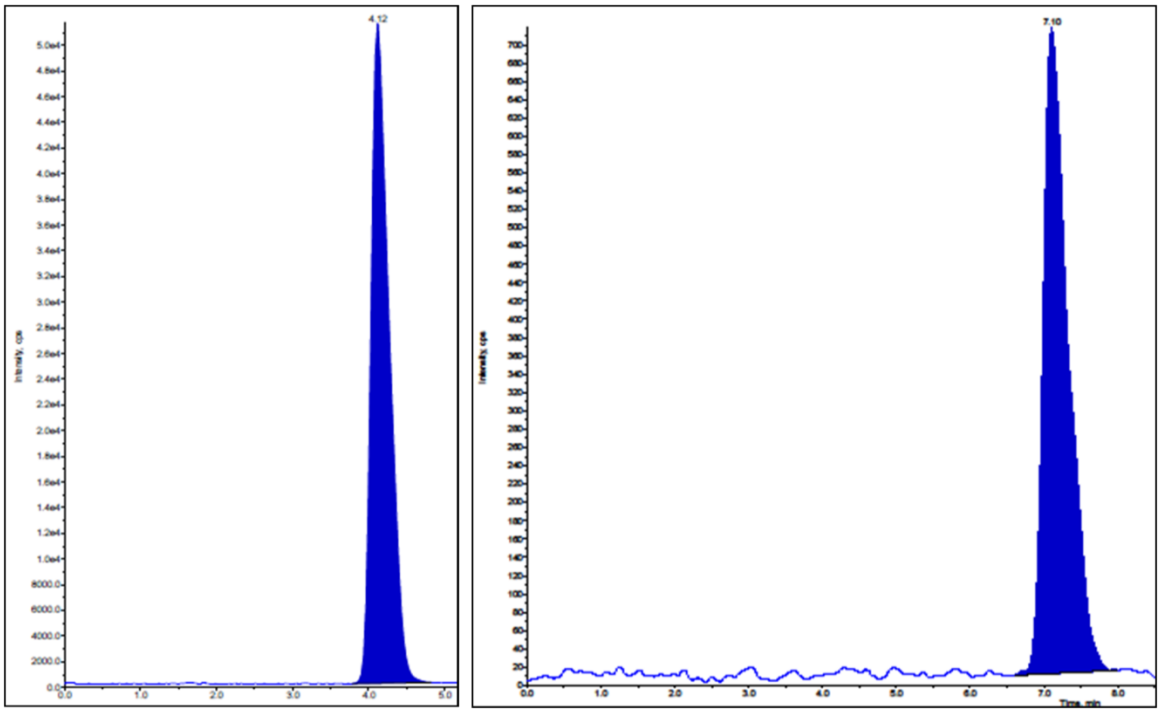


Figure 4.14 Chromatogram for AZ and BD [MIX 3]

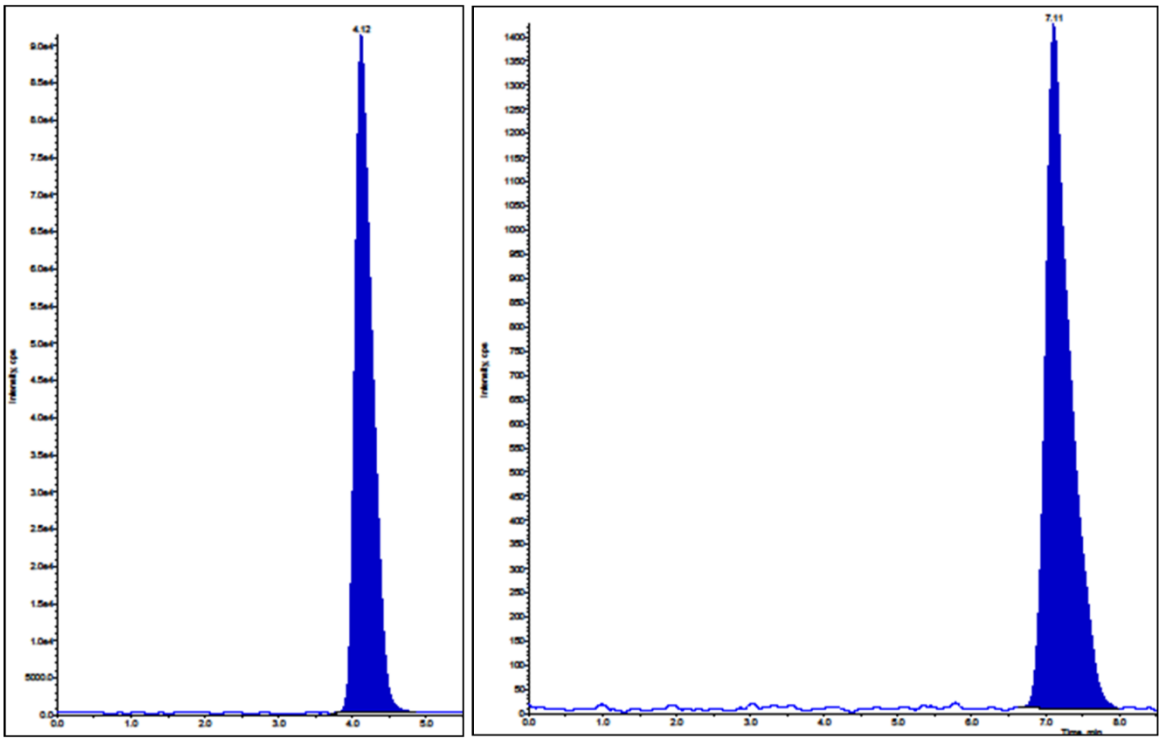


Figure 4.15 Chromatogram for AZ and BD [MIX 4]

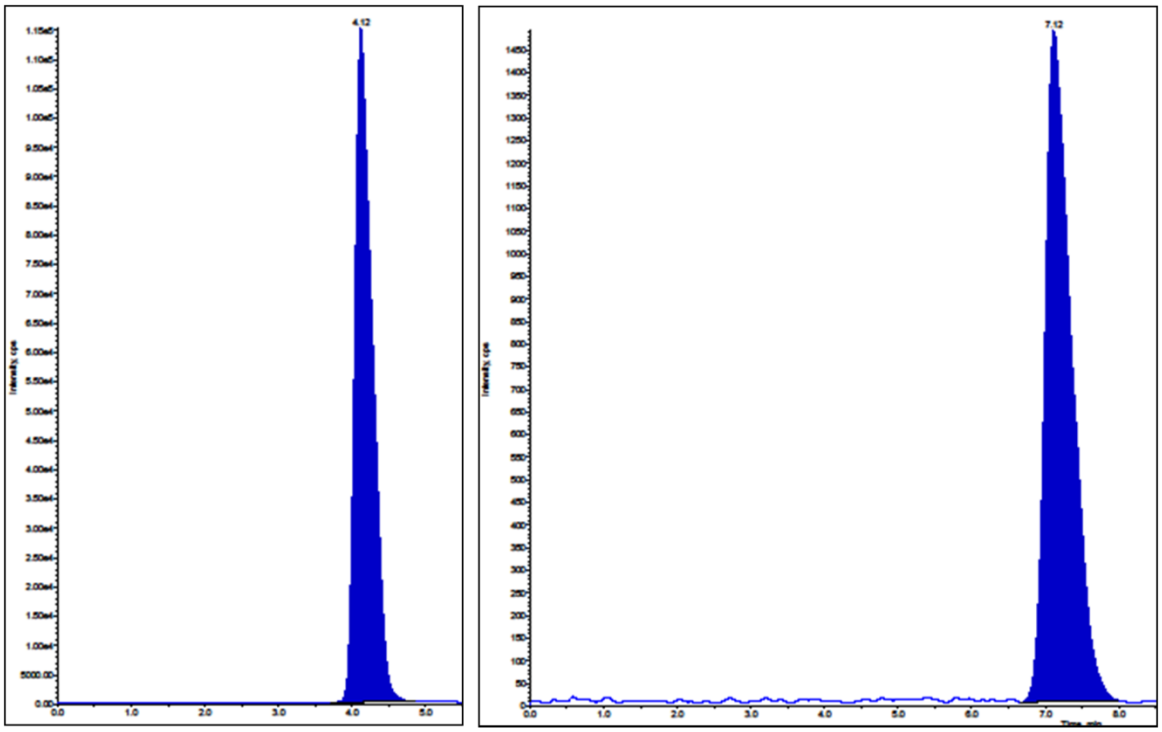


Figure 4.16 Chromatogram for AZ and BD [MIX 5]

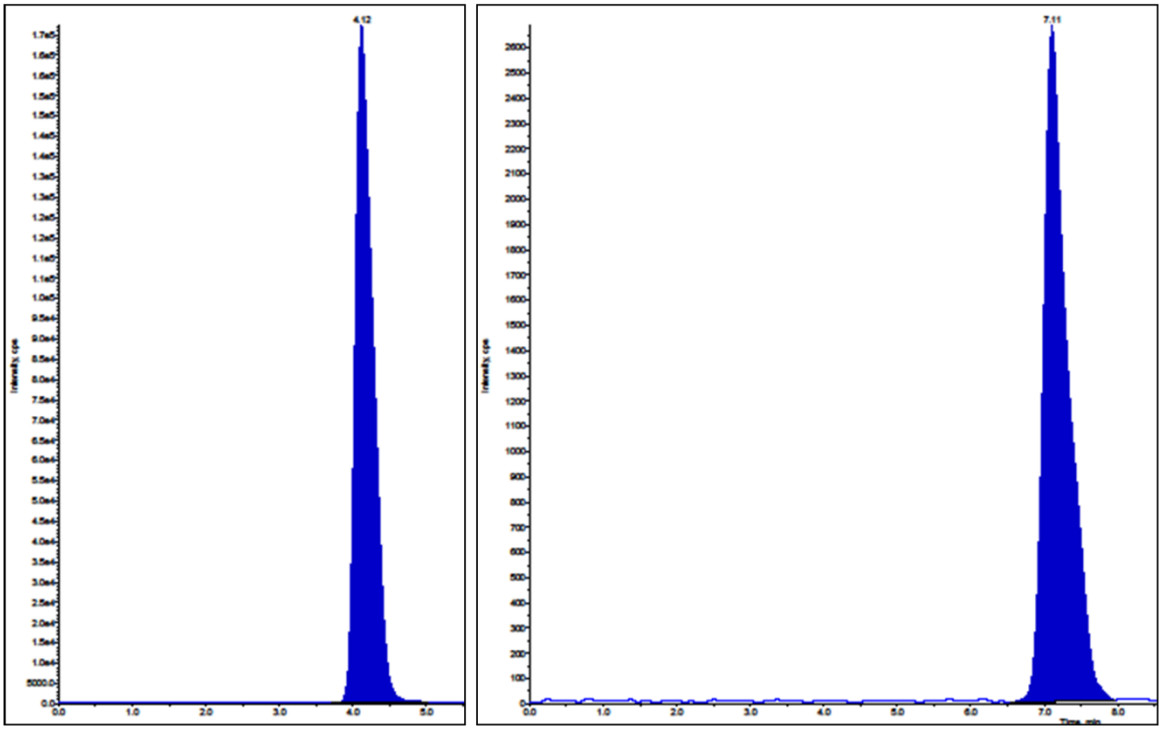


Figure 4.17 Chromatogram for AZ and BD [MIX 6]

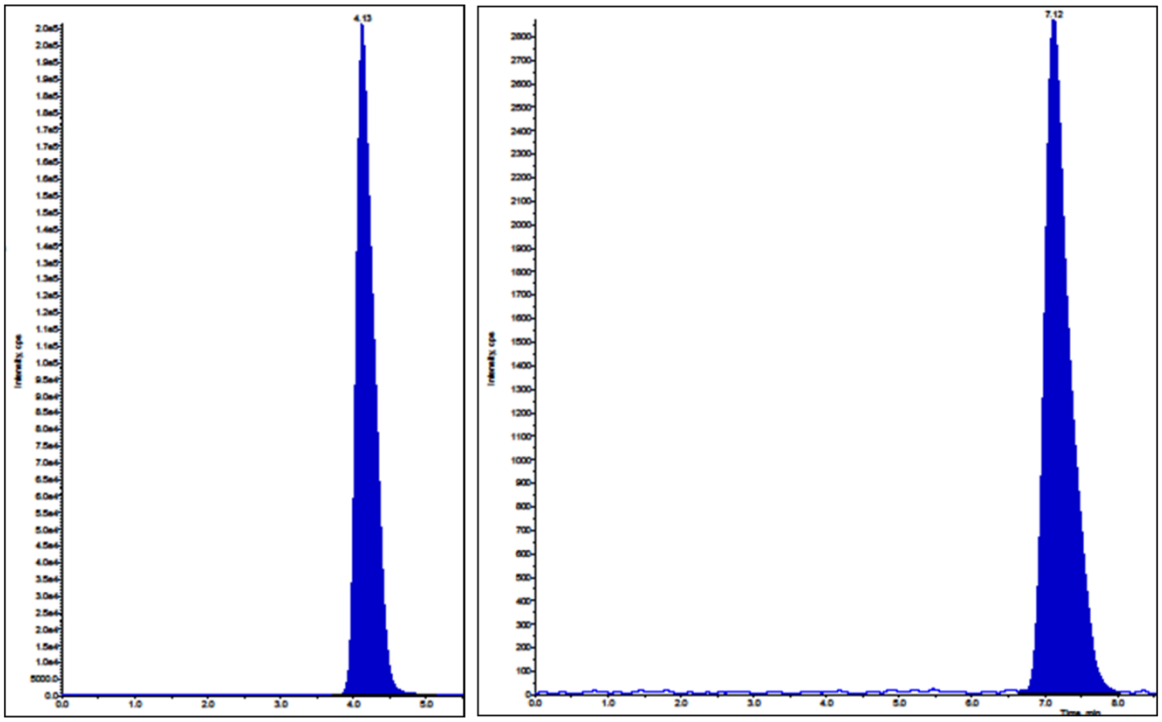


Figure 4.18 Chromatogram for AZ and BD [MIX 7]

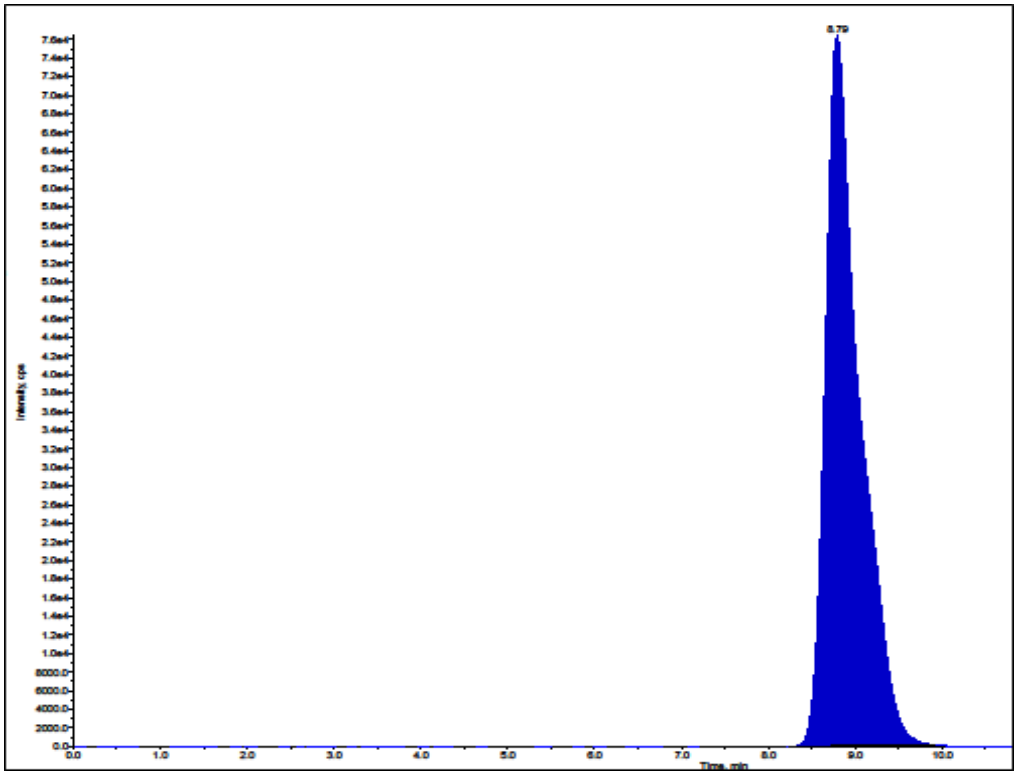


Figure 4.19 Chromatogram for ISTD [MIX 1]

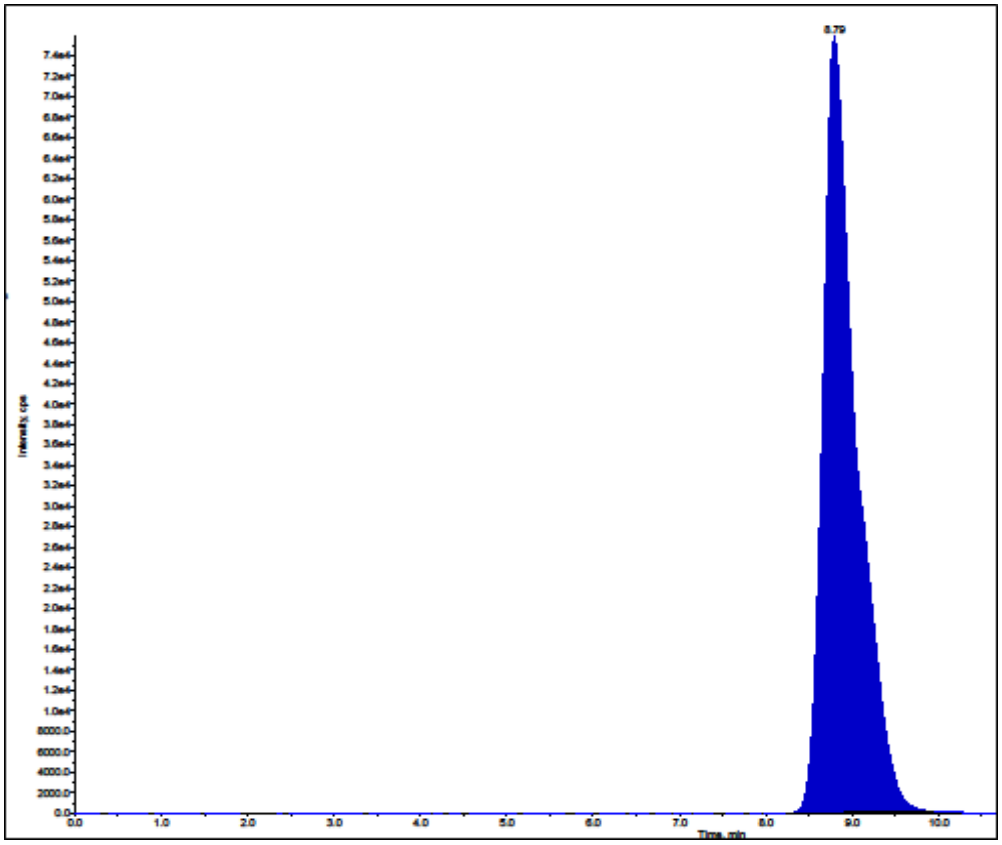


Figure 4.20 Chromatogram for ISTD [MIX 2]

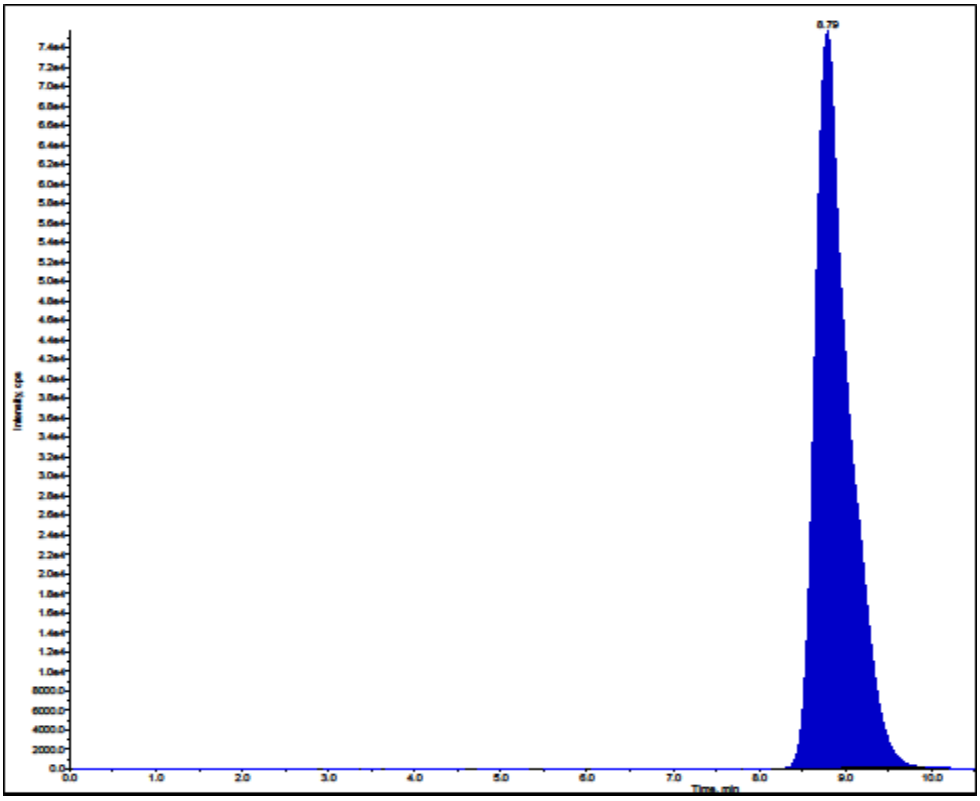


Figure 4.21 Chromatogram for ISTD [MIX 3]

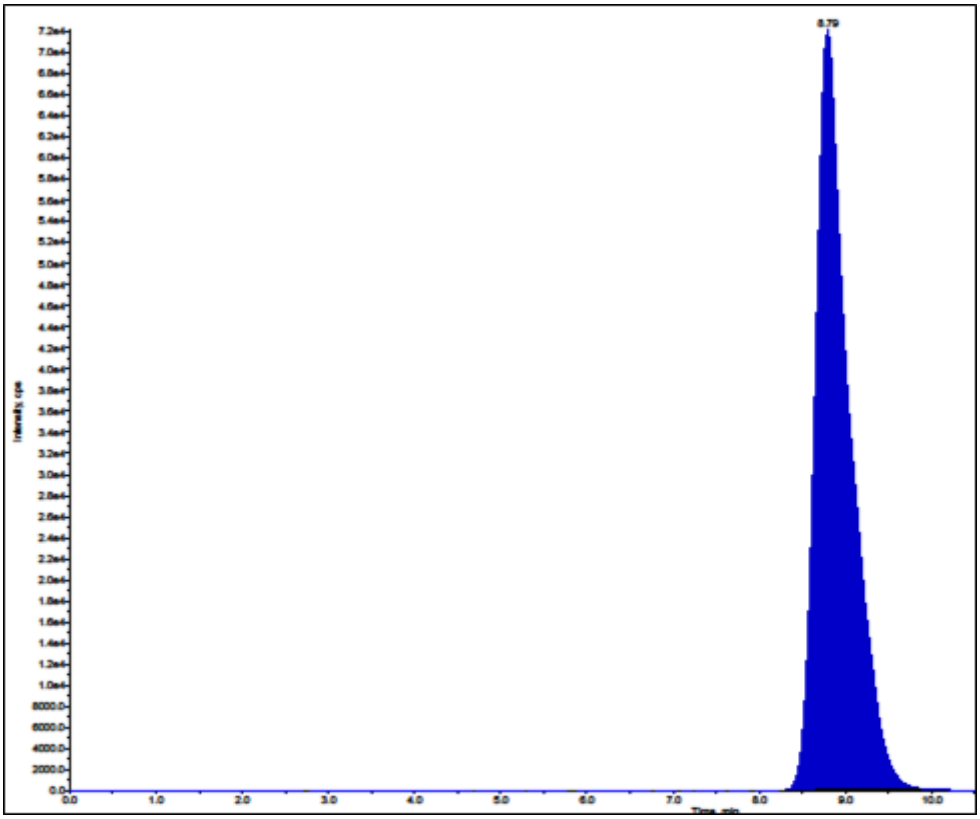


Figure 4.22 Chromatogram for ISTD [MIX 4]

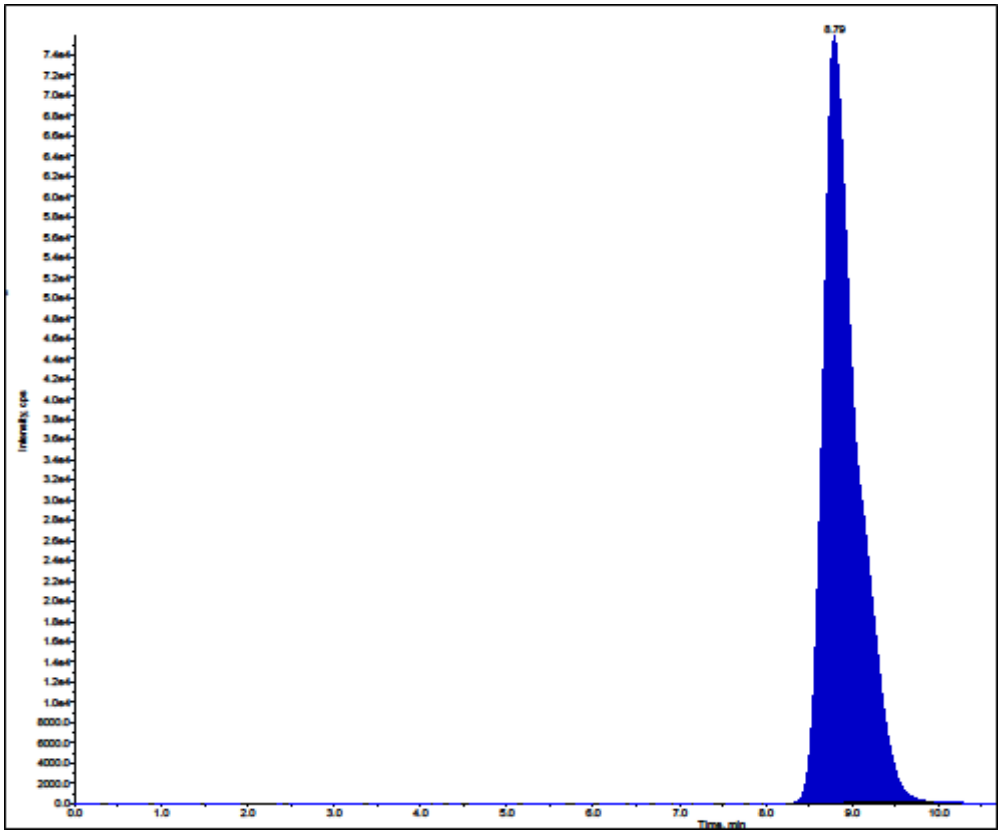


Figure 4.23 Chromatogram for ISTD [MIX 5]

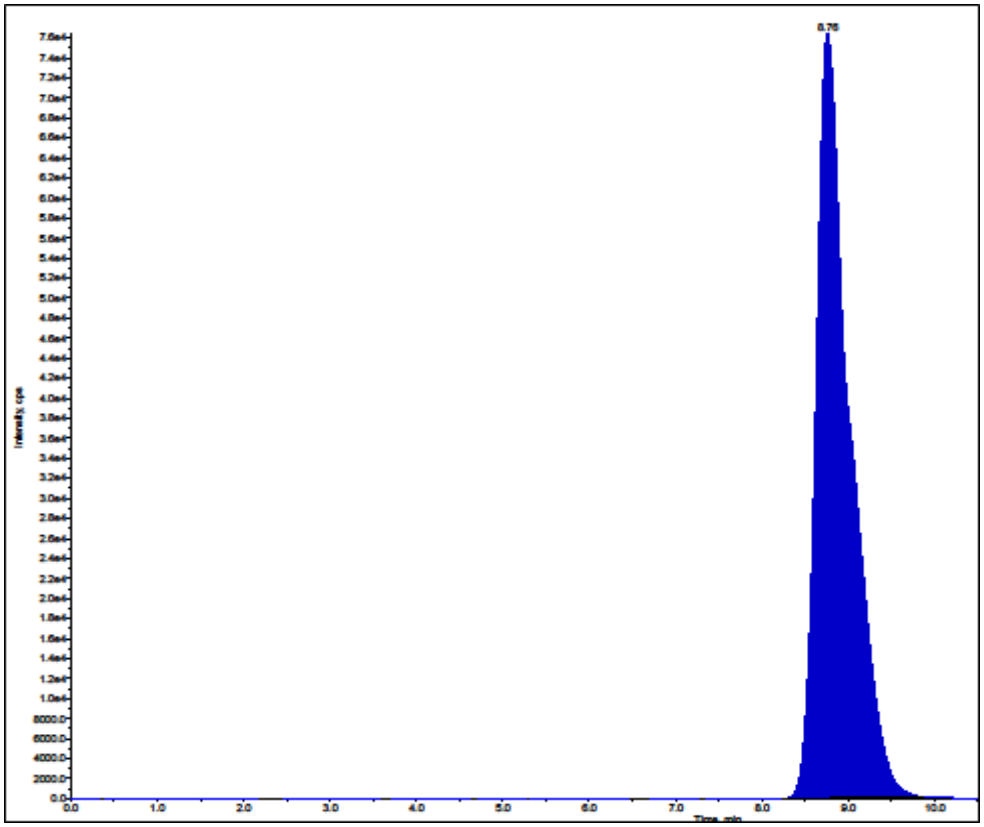


Figure 4.24 Chromatogram for ISTD [MIX 6]

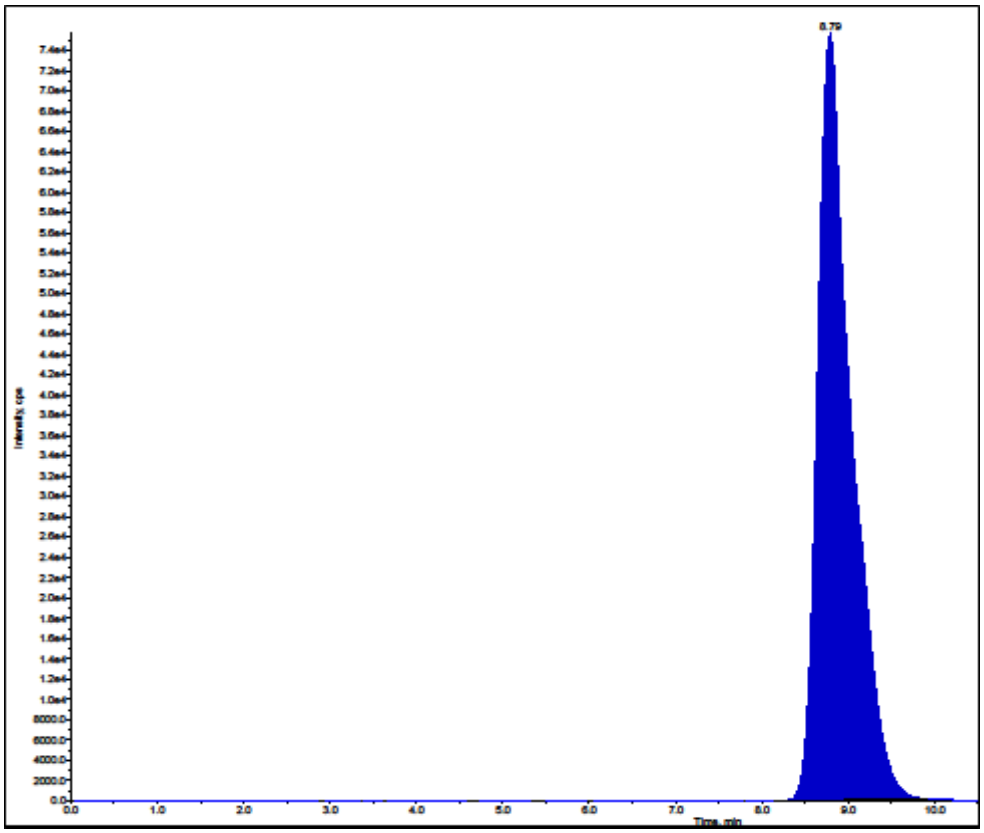


Figure 4.25 Chromatogram for ISTD [MIX 7]

4.4. CONCLUSION

The RP-HPLC method developed earlier (Chapter II, Method 3) by the Box Behnken design was successfully applied for the bioanalytical method development of Azelastine hydrochloride and Budesonide in human plasma. The RP-HPLC method was successfully transferred to the LC-MS/MS and the developed LC-MS method was found to be accurate, precise, sensitive and economical. The two APIs under study could be analysed by the LC-MS method which has a very shorter run time. The method thus developed can be further used for pharmokinetic study and also for estimation of AZ and BD in other biological fluids.

4.5. REFERENCES

1. Elghobashy MR, Badran OM, Salem MY, Kelani KM. Application of Membrane Selective Electrodes for the determination of Azelastine Hydrochloride in the presence of its alkaline degradant in eye drops and plasma. Anal Bioanal Electrochem. 2013; 5(3): 325-40.
2. Pivonka J, Segelman FH, Hartman CA, Segl WE, Kucharczyk N, Sofia RD. Determination of Azelastine and Desmethylazelastine in human plasma by high-performance liquid chromatography. J Chromatogr. 1987; 420: 89-98.
3. Heinemann U, Blaschke G, Knebel N. Simultaneous enantioselective separation of Azelastine and three of its metabolites for the investigation of the enantiomeric metabolism in rats. I. Liquid chromatography–ionspray tandem mass spectrometry and electrokinetic capillary chromatography. J Chromatogr B. 2003; 793: 389–404.
4. Jain L, Doddapaneni S. Clinical Pharmacology Review [Azelastine and Fluticasone]. Dec 2011. Reference ID: 3063275.
   Link: www.fda.gov/downloads/Drugs/.../UCM 304583.pdf. Accessed on: 4 Sept 2015.
5. Park Y, Kim S, Kim Y, Yang S, Lee M, Shaw LM, Kang J. Determination of Azelastine in human plasma by validated liquid chromatography coupled to tandom mass spectrometry (LCESI/ MS/MS) for the clinical studies. Int J Biomed Sci. 2010; 6(2): 120.

6. Zha W, Shum L. Simultaneous determination of azelastine and its major metabolite desmethyl azelastine in human plasma using high performance liquid chromatography–tandem mass spectrometry. J Chromatogr B. 2012; 906: 69-74.
7. Rao TB, Mantha S, Vaidyanathan G. Quantification of Budesonide Using UPLC and Xevo TQ-S. Waters. Application note. Waters India MS Application Laboratory, Bangalore, India.
Link:http://www.waters.com/webassets/cms/library/docs/720004198en.pdf.
Accessed on: 9 Sept 2015.
8. Deventer K, Mikulˇc´ıkov´a P, Hoecke HV, Eenoo PV, Delbeke FT. Detection of budesonide in human urine after inhalation by liquid chromatography–mass spectrometry. J Pharm Biomed Anal. 2006; 42: 474–9.
9. Li YN, Tattam B, Brown KF, Seale JP. Determination of epimers 22R and 22S of budesonide in human plasma by high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J Chromatogr B. 1996; 683: 259-68.
10. Lua Y, Sun Z, Zhang Y, Chen X, Zhong D. Simultaneous quantification of 22R and 22S epimers of budesonide in human plasma by ultrahigh performance liquid chromatography–tandem mass spectrometry: Application in a stereoselective pharmacokinetic study. J Chromatogr B. 2013; 921–922: 27-34.
11. Kronkvist K, Gustavsson M, Wendel AK, Jaegfeldt H. Automated sample preparation for the determination of budesonide in plasma samples by liquid chromatography and tandem mass spectrometry. J Chromatogr A. 1998; 823(12): 401-9.
12. Li YN, Tattama B, Brown KF, Seale JP. Quantification of epimeric budesonide and fluticasone propionate in human plasma by liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry. J Chromatogr B. 2001; 761: 177-85.
13. Borges NCC, Astigarraga RB, Sverdloff CE, Borges BC, Paiva TR, Galvinas PR, Moreno RA. Budesonide quantification by HPLC coupled to atmospheric pressure photoionization (APPI) tandem mass spectrometry. Application to a comparative systemic bioavailability of two budesonide formulations in healthy volunteers. J Chromatogr B. 2011; 879; 236-42.

14. Buschera BAP, Jagfeldt H, Sandman H, Schaik RB, Schaik FV, Brull LP. The determination of budesonide and fluticasone in human sputum samples collected from COPD patients using LC-MS/MS. J Chromatogr B. 2012; 880: 6-11.
15. Kronkvist K, Gustavsson M, Wendel A, Jaegfeldt H. Automated sample preparation for the determination of budesonide in plasma samples by liquid chromatography and tandem mass spectrometry. J Chromatogr A. 1998; 823: 401–9.
16. Krzek J, Czekaj JS, Rzeszutko W, Jo´nczyk A. Direct separation, identification and quantification of epimers 22*R* and 22*S* of budesonide by capillary gas chromatography on a short analytical column with Rtx®-5 stationary phase. J Chromatogr B. 2004: 803: 191-200.
17. Guidance for Industry: Bioanalytical Method validation. US Department of Health and Human Services, 2001, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM) USFDA. 2013.
18. EMEA, "Guideline on validation of bioanalytical methods, EMA committee for medicinal products for human use (CHMP)". 2009.