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# Box-Behnken Design Approach for Optimization of a Liquid Chromatographic Method for the Determination of Anti Leukemic Drugs in Bulk and Pharmaceutical Formulations

**R. Suresh[1*], M. Swamivelmanickam[1] and S. Sivakrishnan[1]**

*[1]Department of Pharmacy, Annamalai University, Annamalainagar-608002, Chidambaram, India.*

***Authors' contributions***

*This work was carried out in collaboration among all authors. Author RS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MS and SS managed the analyses of the study. Author SS managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

To develop and validate a RP-HPLC method for the determination of selected anti- leukemic drugs in bulk and pharmaceutical formulations using Design of experiments. Development of the chromatographic method is based on design of experiments (DOE) approach, utilizing two level full factorial design for screening and Box-Behnken experimental design for optimization. In order to identify the significant parameters for the optimization, by simultaneously registering the main, interaction and quadratic effects of three factors such as volume of methanol ($X_1$), concentration of buffer($X_2$) and flow rate ($X_3$) on the selected responses like capacity factor K1 of first eluted peak ($Y_1$), resolution of critical peaks $R_{S\,(1,2)}$ ($Y_2$) and amp; $R_{S\,(2,3)}$ ($Y_3$) and retention time of last peak $tR_4$ ($Y_4$) as responses. Analytes were separated on a Onyx monolithic- C18 (100×4.6mm) with mobile phase comprising Potassium dihydrogen orthophosphate (0.01M), Methanol and Acetonitrile in ratio of (30:34.29:35.71), with flow rate of 0.723 mL/min and $p^H$ 3.5 adjusted with dilute orthophosphoric acid. Total chromatographic analysis time per samples was approximately 5

**Corresponding author: E-mail: sureshramalingam9@gmail.com;*

minutes with DST(IS), IMT, IBT and SFN eluting with retention times of 2.64, 3.05, 3.81 and 4.59 minutes respectively. Calibration curves were linear over selected range 0.997 for the all analytes. The method was sensitive with the LODs were 10.457, 13.07 and 26.169 ng/mL and LOQs were 31.68, 39.6 and 79.3ng/mL for IMT, IBT and SFN respectively. Inter and Intra-day precision data (in terms of %RSD) was fond to be less than ≥3 respectively, Recoveries ranged ≥102±2% for Imatinib- Gleevec, Ibrutinib- Imbruvica and Sorafenib- Nexavar. The obtained results corroborated the potential of the proposed method for determination of all the three anti-leukemic drugs for routine analysis for products of similar type and composition.

*Keywords: HPLC; Box-behnken design; dasatinib; imatinib; ibrutinib and sorafenib.*

## 1. INTRODUCTION

The deregulation of protein kinase has been identified to play a key role in the molecular pathogenesis of human cancers, such as chronic myelogenous leukemia and also in solid tumors [1]. In Chronic myeloid leukemia (CML) tyrosine kinase receptors are proteins playing an important role in the transduction of the signals involved in growth of cells [2]. The first generation drug used for the treatment of CML is imatinib. The failure of imatinib most likely arises from a combination of tumor and host related factors that contribute to pharmacokinetic variability and induction of resistance [3]. Dasatinib and Nilotinib have revolutionized the treatment of chronic myeloid leukemia and tumors. These drugs are second generation, approximately more potent than imatinib and it also inhibits a number of imatinib-resistant mutant proteins [4].

- Dasatinib (DST), chemical name is N-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl] amino]-5-thiazole carboxamide monohydrate as shown in (Fig. 1a). It is a potent oral inhibitor of multiple oncogenic kinases and used as internal standard.

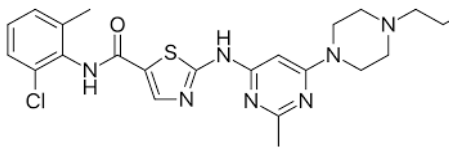

**Fig. 1a. Dasatinib (Internal standard)**

- The chemical name of imatinib mesylate is benzamide, 4-[(-methyl-1-1piperazinyl) methyl]-N-[[4-(3-pyridinyl)-2-pyrimidinyl] amino] phenyl]-, monomethanesulfonate as shown in (Fig. 1b). Imatinib is an antineoplastic agent used to treat chronic myelogenous leukemia and it is protein tyrosine kinase inhibitor that inhibits the Bcr- Abl tyrosine kinase. The constitutive abnormal tyrosine kinase created by the Philadelphia chromosome abnormality in chronic myeloid leukemia.

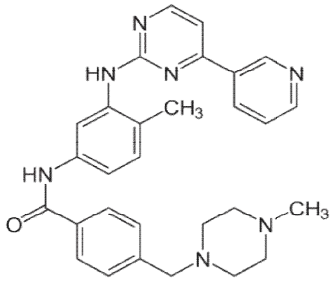

**Fig. 1b. Imatinib**

- Chemical name of ibrutinib is 1-{(3R)-[4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo [3, 4-d] pyrimidin-1-yl] piperdin-1-yl} prop-2-en-1-one as shown in (Fig. 1c). Ibrutinib is a small-molecule inhibitor of Burton's tyrosine kinase (BTK) That targets the ATP binding domain of BTK and forms a covalent bond with a cysteine residue (Cys-481) in the binding pocket that leads to sustained inhibition of BTK enzymatic activity. Ibrutinib is used in the treatment of mantle cell lymphoma (MCL), chronic lymphocytic leukemia, Waldenstroms macroglobulinemia [5].

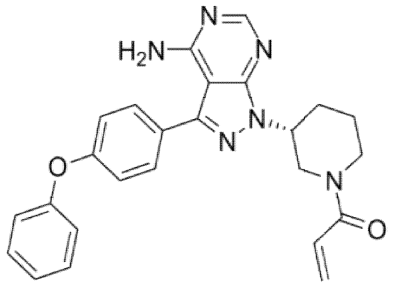

**Fig. 1c. Ibrutinib**

- Chemical name of sorafenib is 4-[4({[4Chloro3 (trifluorométhyl) phenyl] carbamoyl} amino) phénoxy] N-méthyl-2-pyridinecarboxamide as shown in (Fig. 1d) Sorafenib an oral multi- kinase inhibitor is suppressing the activity of several cell surface receptor tyrosine kinases, like vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor and also intracellular members of the mitogen-activated protein kinase (MAPK) signal transduction pathway has been approved for the treatment of advanced renal-cell carcinoma (RCC) and hepatocellular carcinoma(HCC) [6,7].

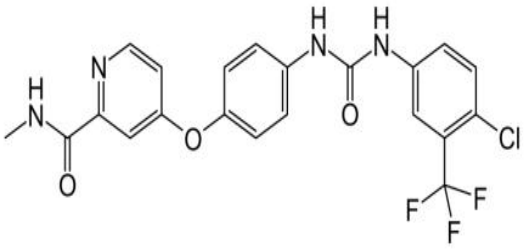

**Fig. 1d. Sorafenib**

The Rational behind the selection of these anti leukemic drugs is that in cancer patients, the novel treatment regimen includes either any one of these drugs or in combinations with other TKIs. A typical combination is specific to individuals suffering from cancer this specificity of selecting combination of drugs (TKIs) mainly based on the genomics or gene coding that is specific to individuals. In recent years, there have been many reports on individual determination of selected (TKIs) in pharmaceuticals and biological matrices and additionally many liquid chromatographic methods are reported for the simultaneous determination of IMT, IBT and SFN tyrosine kinase inhibitors (TKI's) [8-17]. Furthermore, most of these methods suffer from limitations such as poor retention, complicated procedure, expensive use of solvents and instrumentation to achieve better chromatographic separation and low detection capability for estimation in formulations. Till date, there are no methods reported in the literature mentioning the use of design of expert (DOE's) for the development, optimization and validation of HPLC methods or LC-MS methods for the analysis of these TKI's. Hence in our present research work we have employed BBD, as a DOE tool to optimize the chromatographic conditions for the determination of IMT, IBT and SFN in APIs and pharmaceutical dosage forms.

Two-level full and factorial designs were used to screen the important factors that influence process output measures and product quality. A two-level full factorial design consists of two level of each experimental factor and has design matrix made up of all combinations of these factors levels. It can provide the direction for further experimentation. Response surface methodology (RSM) is statistical technique used for the development and optimization of complex process [18,19]. RSM is used after preliminary screening of experimental factors that significantly affect the response using factorial design. The technique has several advantages over conventional optimization method in which one variable is used at a time. RSM provides a large amount of information and is a relatively economical approach because a small number of experiments are performed for monitoring the interaction of the independent variables and the response. In conventional optimization, the increase in the number of experiments necessary to carry out the research, leads to an increase in time and expenses as well as in the utilization of reagents and materials for experiments [20].

Many types of response surface designs are used for optimization, such as central composite, Doehlert and Box-Behnken designs. The Box-Behnken design is preferable to central composite and Doehlert designs because it requires fewer test runs and is rotatable. The Box-Behnken design is advantageous because it does not contain any points at the extremes of the cubic region created by the two- level factorial combinations [21,22]. Hence here in our research work we here developed a novel RP-HPLC method for the simultaneous determination of DST, IMT, IBT and SFN (TKIs) by utilizing BBD. This developed method can be utilized for the quantitative analysis of any one these drugs alone or in combination along with other TKIs drugs. This developed method could also be applied for TDM, Bio equivalence studies and drug-drug interaction studies.

## 2. EXPERIMENTAL DESIGN

### 2.1 Instrumentation

The chromatographic method development and validation was performed on Shimadzu HPLC (Shimadzu Corporation, Kyoto, Japan). The

system consisted of two LC-20AD solvent delivery modules: an SPD-M 20A PDA detector and a Rheodyne injector (model 7125, USA) valve fitted with a 20 µL loop. Chromatographic data were collected and processed using LC solutions® software (Version 1.11SP1). The mobile phase was degassed using Branson sonicator (Branson Ultrasonic, USA).

## 2.2 Chromatographic Condition

The chromatographic separation was carried out using a mobile phase consisting of a mixture of potassium dihydrogen orthophosphate (aqueous content), MeOH and MeCN. The analytes were detected at 264 nm based on isobestic point. Prior to use, the mobile phase was degassed for 10 min in ultrasonic bath and vacuum filtered through 0.45µm membrane filter (Gelman Science, India). The mobile phase was prepared by mixing appropriate proportions of aqueous content with MeOH and MeCN to the mixture as per design. The HPLC system was used at an ambient temperature (25±2°C).

## 2.3 Chemicals and Reagents

The working standards of Dasatinib (IS), Bristol-Myers Squibb India Pvt. Ltd., (DST) India, Imatinib, Novartis India [IMT], Ibrutinib, Janssen Biotech Pvt. Ltd., [IBT], and Sorafenib, Bayer pharmaceuticals Pvt. Ltd., were donated by respected manufacturers. Methanol and Acetonitrile was HPLC grade, Orthophosphoric acid and $KH_2PO_4$ of analytical grade was purchased from SD fine Chemicals, Mumbai, India. High purity HPLC grade water was prepared by using Milli-Q Academic, Millipore (Bangalore, India).

## 2.4 Design of experiments

Design of experiments, data analysis and Derringer desirability function calculations were performed by using Design expert®, 11.0.2.0 version. The rest of the calculations were performed using the Microsoft excel 2010 software.

## 2.5 Stock and working Standard Solutions

Stock standard solutions of Dasatinib (Internal standard), Imatinib, Ibrutinib and Sorafenib, at 1000µg/mL, were prepared individually using mixture of MeOH and water in 80:20 v/v and stored at 4°C protected from light. The solutions of DST(IS), IMT, IBT and SFN further diluted with the mobile phase to give a series of standard mixtures having a final concentration range 2-25µg/mL. The solution prepared for the optimization procedure comprised of DST, IMT, IBT and SFN, at10µg/mL.

## 2.6 Preparation of the Sample Solution

Ten tablets of and Ten capsules of were Imatinib- Gleevec, Ibrutinib- Imbruvica and Sorafenib- Nexavar. Weighed and analyzed separately. An amount of powder equivalent to 10 mg was weighed and transferred in a 10mL volumetric flask, and 5mL of mobile phase was added. This mixture was subjected to sonication for approximately 15 min to ensure complete solubility of drugs, and the solution was made up to the mark with mobile phase and further dilutions were made to obtain concentration of IMT 8.0µg/mL, IBT 10µg/mL and SFN 20µg/mL The resulted solutions were centrifuged at 4000 rpm for 10 min, and clear supernatant was collected and filtered through a 0.2µm membrane filter (Gelman Sciences, India). A 20µl of the final solution was injected in triplicate and chromatographed.

## 2.7 Validation

Validation studies were conducted using the optimized assay conditions based on the principles of validation described in the ICH guidelines “Text on validation of Analytical Procedures [23]” and “Q2B, Validation of Analytical Procedure: Methodology [24]”. Key analytical parameters, including, accuracy, precision, linearity, detection limit, quantitation limit was evaluated.

# 3. RESULTS AND DISCUSSION

A RP-HPLC method was developed, optimized and validated for determining the various TKI’s namely DST, IMT, IBT and SFN. The quality by design approach using FFD and BBD was utilized for screening and optimizing the chromatographic separation problems due to interferences between drugs, excipients and solvent components.

## 3.1 Initial Screening

Further screening of the Qbd was performed using FFD. Prior to method development, a set of

preliminary experiments were performed using different compositions of water, potassium dihydrogen orthophosphate, MeOH and MeCN. Under these conditions, TKI's peaks were co-eluted and did not result in good separation. The resolution between Dasatinib and Imatinib $R_{S\ (1,2)}$ and Imatinib and Ibrutinib $R_{S\ (2,3)}$ peaks are very critical to separation. This might to be attributed to poor of affinity of the TKI's to the monolithic C-18.

## 3.2 Screening Design by Two- level FFD

Screening designs are normally used when large numbers of factors are likely to affect a particular response[25]. A two level full factorial design was employed to evaluate the effect of three independent factors, namely MeOH ( $X_1$), buffer ($X_2$) and flow rate ($X_3$) on responses capacity factor $K^1$ of first eluted peak ($Y_1$), resolution of critical peaks $R_{S\ (1,2)}$ ($Y_2$) & $R_{S\ (2,3)}$ ($Y_3$) and retention time of last peak $tR_4$ ($Y_4$) . On the basis of preliminary experiments the range of values used in the design are the following: MeOH ($X_1$) 25-35 v/v; buffer ($X_2$) 0.005-0.01Mm and flow rate ($X_3$) 0.6-0.9mL/min. All the experimental runs were performed in triplicate and results design matrix of the two-level full factorial design is provided in (Table 1).

## 3.3 Optimization by Response Surface Methodology-BBD

A three- level Box- Behnken design with six center points (Table 2) was to evaluate the main, interaction and quadratic effects of MeOH ( $X_1$), buffer ($X_2$) and flow rate ($X_3$) on responses capacity factor $K^1$ of first eluted peak ($Y_1$), resolution of critical peaks $R_{S\ (1,\ 2)}$ ($Y_2$) & $R_{S\ (2,\ 3)}$ ($Y_3$) and retention time of last peak $tR_4$ ($Y_4$) .The experimental conditions that were maintained constant include the $KH_2PO_4$ (pH 3.6, 30mL). All the experimental runs were performed in triplicate and results are presented in (Table 2). The screening FFD, aided in selection of factors and ranges that could influence the quality of separation of the analytes viz. % aqueous ($X_1$:25-35 v/v), Buffer ($X_2$:0.005-0.01Mm) and Flow rate ($X_3$:0.6-0.9mL/min). In order to judge the quality of the chromatographic separation, under different conditions, the following responses were defined viz. retention factor of first eluted peak ($k^1$) ($Y_1$), ,resolution between first two peaks $R_{S\ (1,\ 2)}$ ($Y_2$), resolution between second and third peaks $R_{S\ (2,\ 3)}$ ($Y_3$), and retention time of last eluting peak $tR_4$ ($Y_4$). To arrive at a decision between selectivity and analysis time, Box-Behnken design was used in current chromatographic analysis. Three factors generated a total number of 18 runs including 6 center points (Table 2).

**Table 1. Full factorial design (FFD)**

| S. No | Factor levels | | | $X_4$ | Responses | | | |
|---|---|---|---|---|---|---|---|---|
| | $X_1$ MeOH% v/v | $X_2$ Buffer Concn. | $X_3$ Flow rate mL $min^{-1}$ | pH | $K_1$ ($Y_1$) | $Rs_{(1,2)}$ ($Y_2$) | $Rs_{(2,3)}$ ($Y_3$) | $tR_4$($Y_4$) |
| 1 | 25 | 0.005 | 0.6 | 3.2 | 0.444 | 2.499 | 0 | 4.870 |
| 2 | 25 | 0.1 | 0.9 | 3.2 | 0.263 | 1.116 | 2.240 | 3.503 |
| 3 | 35 | 0.005 | 0.6 | 3.2 | 0.465 | 1.872 | 0.782 | 5.555 |
| 4 | 35 | 0.01 | 0.9 | 3.6 | 0.344 | 1.359 | 3.111 | 4.371 |
| 5 | 35 | 0.01 | 0.6 | 3.2 | 0.288 | 1.299 | 2.68 | 5.51 |
| 6 | 25 | 0.005 | 0.9 | 3.2 | 0.434 | 2.110 | 0 | 3.226 |
| 7 | 25 | 0.005 | 0.6 | 3.6 | 0.489 | 2.580 | 0 | 5.163 |
| 8 | 35 | 0.005 | 0.9 | 3.6 | 0.465 | 1.767 | 0.882 | 3.921 |
| 9 | 25 | 0.01 | 0.6 | 3.6 | 0.303 | 1.322 | 1.203 | 4.820 |
| 10 | 25 | 0.01 | 0.6 | 3.2 | 0.274 | 1.181 | 2.414 | 5.286 |
| 11 | 25 | 0.005 | 0.9 | 3.6 | 0.477 | 2.161 | 0 | 3.300 |
| 12 | 35 | 0.005 | 0.9 | 3.2 | 0.458 | 1.562 | 0.701 | 3.66 |
| 13 | 25 | 0.01 | 0.9 | 3.6 | 0.296 | 1.280 | 1.168 | 3.183 |
| 14 | 35 | 0.01 | 0.9 | 3.2 | 0.286 | 1.197 | 2.493 | 3.676 |
| 15 | 35 | 0.01 | 0.6 | 3.6 | 0.347 | 1.543 | 3.370 | 6.57 |
| 16 | 35 | 0.005 | 0.6 | 3.6 | 0.469 | 1.878 | 0.890 | 5.846 |

**Table 2. Box-Behnken Design (BBD)**

| S. No | Factor levels | | | Responses | | | |
|---|---|---|---|---|---|---|---|
| | *A* MeOH % v/v | *B* Buffer | *C* Flow rate mL $min^{-1}$ | $K_1$ | $Rs_{(1,2)}$ | $Rs_{(2,3)}$ | $tR_4$ |
| 1 | 30 | 0.01 | 0.9 | 0.266 | 1.203 | 2.686 | 3.57 |
| 2 | 35 | 0.005 | 0.75 | 0.536 | 2.942 | 0 | 4.396 |
| 3 | 35 | 0.0075 | 0.6 | 0.387 | 1.745 | 1.367 | 5.39 |
| 4 | 35 | 0.0075 | 0.9 | 0.394 | 1.68 | 1.337 | 3.781 |
| 5 | 30 | 0.0075 | 0.75 | 0.396 | 1.81 | 0.952 | 4.291 |
| 6 | 30 | 0.01 | 0.6 | 0.293 | 1.287 | 2.13 | 5.1 |
| 7 | 30 | 0.005 | 0.6 | 0.487 | 2.82 | 0 | 5.34 |
| 8 | 30 | 0.0075 | 0.75 | 0.415 | 1.83 | 0.954 | 4.295 |
| 9 | 25 | 0.005 | 0.75 | 0.459 | 2.199 | 0 | 3.844 |
| 10 | 30 | 0.0075 | 0.75 | 0.396 | 1.81 | 0.952 | 4.291 |
| 11 | 35 | 0.01 | 0.75 | 0.303 | 1.398 | 2.547 | 4.462 |
| 12 | 30 | 0.0075 | 0.75 | 0.415 | 1.83 | 0.959 | 4.295 |
| 13 | 30 | 0.005 | 0.9 | 0.485 | 2.606 | 0 | 3.554 |
| 14 | 30 | 0.0075 | 0.75 | 0.396 | 1.81 | 0.952 | 4.291 |
| 15 | 25 | 0.0075 | 0.9 | 0.368 | 1.546 | 0.862 | 3.464 |
| 16 | 25 | 0.0075 | 0.6 | 0.366 | 1.781 | 0.934 | 5.167 |
| 17 | 25 | 0.01 | 0.75 | 0.279 | 1.284 | 1.866 | 4.04 |
| 18 | 30 | 0.0075 | 0.75 | 0.415 | 1.83 | 0.959 | 4.295 |

All experiments were performed in duplicate and randomized order, to assess the experimental error and to test the predictive validity of the model [26]. A cross effect between the three factors ($X_1$, $X_2$ and $X_3$) and the obtained responses were then evaluated using a "Standard least squares" model. To obtain a simple and yet a realistic model, the insignificant terms ($p > 0.05$) were excluded from the model through a 'backward elimination process. The statistical parameters obtained for reduced models by ANOVA are presented in (Table 3).

The adjusted $R^2$ was well within the acceptable limits of $R^2 \geq 0.9492$, revealing that the experimental data showed a good fit with the second order polynomial equations [27]. The adequate precision is measure of the "signal (response) to noise (deviation) ratio. Usually, a ratio greater than 4 is desirable. The adequate precision values were found to be in the range of 19-36, indicating an adequate signal, and therefore, the model is significant for the chromatographic separation [28]. The CV% for all models was found to be less than 10%, signifying that the model is consistent and

**Table 3. Response models[a] and statistical parameters obtained from ANOVA for CCD**

| Responses | Response models | Adjusted $R^2$ | Model *P* value | %C.V | Adequate precision |
|---|---|---|---|---|---|
| $K_1$ | K1 =+0.4055 +0.0185 A -0.1033B 0.0025 C -0.0132 AB +0.0013AC -0.0062 BC -0.0076 $A^2$-0.0036$B^2$ -0.0191 $C^2$ | 0.9750 | <0.0001 | 2.97 | 29.7596 |
| $Rs_{(1,2)}$ | Rs(1,2) =+0.199125 +0.262025 MeOH -469.45000 Buffer +0.776667 FR -12.58000 MeOH * Buffer +0.056667 MeOH * FR +86.66667 Buffer * FR -0.003105 $MeOH^2$ +34140.00000 $Buffer^2$ -2.41667 $FR^2$ | 0.9492 | <0.0001 | 6.05 | 19.8549 |
| $Rs_{(2,3)}$ | Rs(2,3) =+0.9555 +0.1986 A +1.15 B+0.0568 C +0.1702 AB +0.0105 AC +0.1390 BC +0.0344 $A^2$ +0.1134$B^2$ +0.1351 $C^2$ | 0.9849 | <0.0001 | 9.30 | 36.0742 |
| $tR_4$ | tR4 = +4.29 +0.1905 A +0.0060B -0.8285 C -0.0350 AB +0.0235AC +0.0640 BC -0.0252 $A^2$ -0.0847$B^2$ +0.1827 $C^2$ | 0.9805 | <0.0001 | 1.92 | 32.8513 |

suitable for prediction of optimization. The targeted criteria for optimization and weight, importance for all the factors were shown in (Table 4). As can be seen in (Table 3), the interaction term with the largest absolute coefficient among the fitted models are significant and the responses k1, $Rs_{(1,2)}$, $Rs_{(2,3)}$ and tR4 the largest absolute coefficient among the fitted models are AC (0.0013), *AC* (+ 0.0425), AB(+0.1702) and BC(+0.0640) respectively. The study reveals that changing the fraction of MeOH from low to high results in a rapid decline in the retention time of DST (IS), IMT, IBT and SFN. Therefore, when the MeOH concentration has to be at its highest level to shorten the run time.

Especially this interaction is synergistic, as it leads to a decrease in run time. In (Fig. 2) prediction profiler plots are presented for predicted models in order to gain a effect of individual factors on a specific response. The advantage of the prediction profiler over interaction plots is that it shows all the effects of individual factors on all the responses in a single window.

A steepest slope or curvature indicates sensitiveness of the response to a specific factor. The criteria for the optimization of individual responses are shown in (Table 4).

The highest desirability value of 0.916 was achieved at aqueous content (MeOH) of 34.249% v/v, concentration of buffer is 0.010mM and flow rate of 0.723mL/min. The predicted response values corresponding to higher desirability were $k_{1=}$ 0.319; $R_{S\ (1,\ 2)\ =}$1.343; $R_{S\ (2,\ 3)}$ = 2.275 and $tR_4$=4.5 min. The prediction efficiency of the model was tested by performing the experiment under optimal conditions and obtained chromatogram is shown in (Fig. 3) and results were also supported by overlay plot in (Fig. 4). The average errors for $k_1$, $R_{S\ (1,\ 2),}$ $R_{S\ (2,\ 3)}$ and $tR_4$ were 2.19, 3.27, 3.780 and 2 respectively. The average error values were <4%, indicating a good agreement between the experimental and the predicted responses.

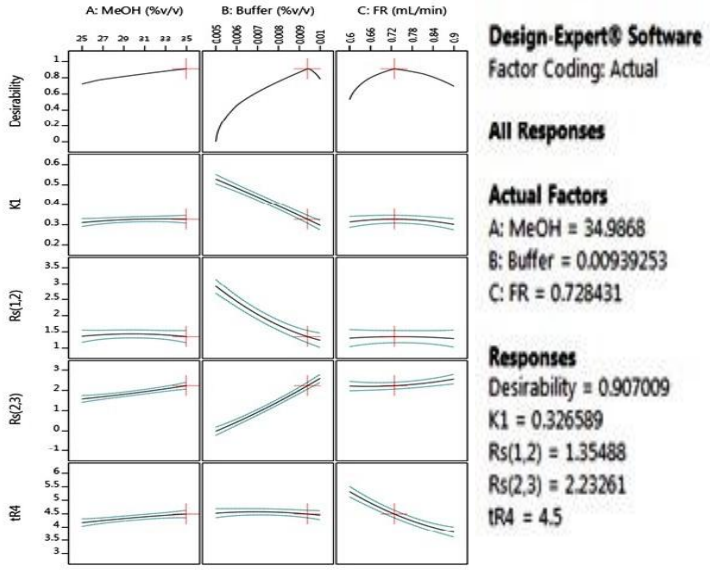


**Fig. 2. Predicition profiler obtained for the responses $K_1$, $Rs_{(1,2)}$, $Rs_{(2,3)}$ and $tR_4$**

**Table 4. Criteria for the optimization of the individual responses for the analysis of quality control samples**

| Responses | Lower limit | Upper limit | Criteria I | | |
|---|---|---|---|---|---|
| | | | Goal | Importance | weights |
| $K_1$ | 0.266 | 0.536 | In range | 3 | 1 |
| $Rs_{(1,2)}$ | 1.203 | 2.942 | In range | 2 | 1 |
| $Rs_{(2,3)}$ | 0 | 2.686 | maximize | 5 | 1 |
| $tR_4$ | 3.464 | 5.39 | Target= 4.5 | 2 | 1 |

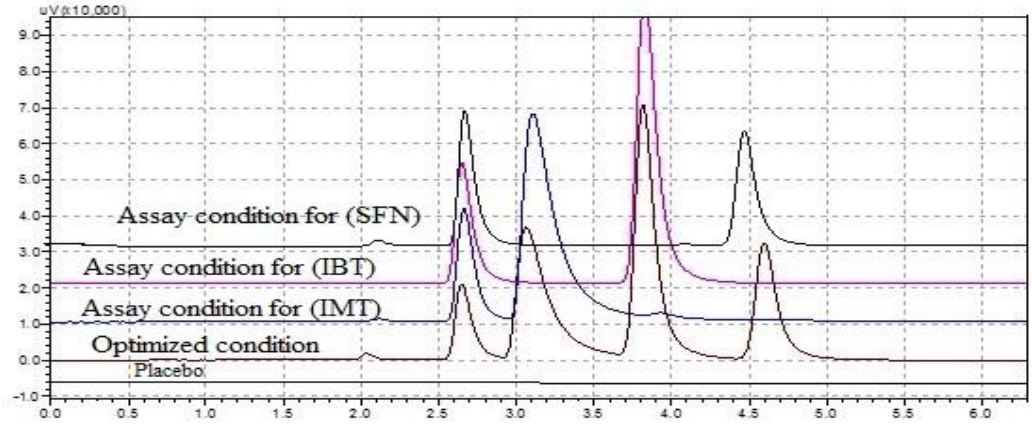


**Fig. 3. Representative overlaid Chromatograms corresponding to (a) Placebo solution; (b) optimized condition (c) Assay condition for IMT 8µg/ml (d) Assay condition for IBT 10µg/ml (e) Assay condition for SFN 20µg/ml with IS under optimal conditions**

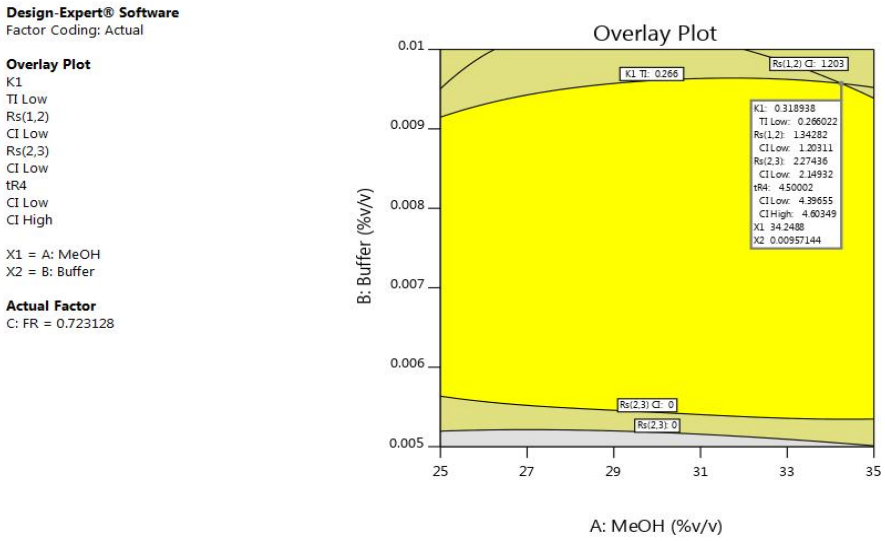


**Fig. 4. Overlay plot supported by all responses**

## 3.4 Assay Method Validation

The last step of the study was to check method validation for specificity, linearity, intra/inter-day precision, and robustness. The optimized HPLC method was specific in relation to the placebo used in the study. All placebo chromatograms showed no interference peaks (Fig. 4). An excellent linearity was established at five level in the range of 2-25 µg/ml for IMT,IBT and SFN and 7µg/ml of DST(IS) with $R^2$ of more than 0.99 for all the analytes. The slope and intercept of the calibration curve were0.521 and + 0.131 for IMT and 0.510 and +0.211 for IBT and 0.100 and +0.279 for SFN respectively. Since the correlation coefficients are not good indicators of linearity performance of an analytical procedure a one-way ANOVA was performed. For all the analytes, the calculated F- Value (F calc) was found to be less than the theoretical F-value (F *crit*) at 5% significance level, indicating that there was no significance difference between replicate determinations for each concentration level. The LODs were 10.457, 13.07 and 26.169 ng/mL and LOQs were 31.68, 39.6 and 79.3ng/mL for IMT, IBT and SFN respectively. Accuracy (n=9), assessed by spike recovery, were found to be 102.47, 99.8 and 101.7 for IMT,IBT& SFN respectively, with were within acceptable ranges of 100± 2% [34]. The intra and inter-assay

precision (n=6) was confirmed since, the %CV were well within the target criterion of ≥2 and ≤ 3, respectively. Robustness study reveals that small changes did not alter the retention times, retention factor, and resolution and therefore it would be concluded that the method conditions are robust.

## 4. CONCLUSION

A two–level full factorial design was used to design an experimental program to provide data regarding influencing factors that had significant effects on the selected responses. One-way ANOVA and Pareto ranking analyses showed that the volume of methanol ($X_1$), concentration of buffer($X_2$), pH ($X_3$) and flow rate ($X_4$) were statistically significant factors($P<0.005$. A Box-Behnken experimental design with response surface methodology was then utilized to evaluate the main, interaction and quadratic effects of these three factors on the selected responses like capacity factor $K^1$ of first eluted peak ($Y_1$), resolution of critical peaks $R_{S\,(1,2)}$ ($Y_2$) & $R_{S\,(2,3)}$ ($Y_3$) and retention time of last peak $tR_4$ ($Y_4$) .With the help of the response surface plots prediction profiler and over lay plot, values the optimum values of the selected factors were identified. It was concluded that the experimental design concept could be effectively used to optimize a high performance liquid chromatographic method for the determination of selected anti leukemic drugs in bulk and pharmaceutical formulations with the least number of experimental runs possible.

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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