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Chemometric Optimization for Simultaneous Determination of Anti-retroviral Drugs by RP-HPLC

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ABSTRACT

This work deals with multiple response simultaneous optimization using the Derringer's desirability function for the development of a reversed phase HPLC method for the simultaneous determination of lamivudine, tenofovir and efavirenz in commercial pharmaceutical preparations. Twenty experiments, taking the capacity factor of the first peak, resolution between the secondand third peaks and the retention time of the thirdpeak as the responses with three important variables as organic phase composition, buffer molarity, and flow rate, were used to design mathematical models. The experimental responses were fitted into a second order polynomial and the three responses were simultaneously optimized to predict the optimum conditions for the effective separation of the studied compounds. The optimum assay conditions were: methanol–triethylamine buffer (pH 3.0; 15.3 mM) (35:65%v/v) as the mobile phase and at a flow rate of 1.19 ml/min. While using this optimum condition, a baseline separation with a minimum resolution of 2.0 and a run time of less than 6 min wasachieved. The method showed a good agreement between the experimental data and predictive value throughout the studied parameter space. The optimized assay condition was validated according to the International Conference on Harmonization guidelines to confirm specificity, linearity, accuracy, and precision.

Keywords: multiple response optimization; Derringer's desirability function; reversed-phase HPLC; central composite design; lamivudine, tenofovir disoproxil fumerate, efavirenz..

INTRODUCTION

Chromatographic analysis usually involves three steps. They are sample preparation, compound separation, and compound quantification. Of these, the steps of sample preparation and compound separation have been frequently optimized employing multivariate statistical techniques.[1]Most analytical procedures attempt for a univariate process. Abnormality cannot be seen when considering only one variable at a time. Such problems are more complex by nature. These complex problems can be solved by multivariate techniques - meaning that there are many variables that contribute to them. Multivariate analysis is a statistical technique used to find patterns and relationships between several variables simultaneously**.** It lets us predict the effect; a change in one variable will have on other variables.Since HPLC utilizes a wide selection of chromatographic factors, namely, the type and concentration of organic modifier, pH, buffer molarity, temperature, flow rate, etc., optimization of the experimental conditions is a complicated process. Therefore, a systematic approach such as an experimental design to optimize chromatographic separations is more essential.[2,3]The best experimental design approach for the purpose of modeling and optimization isthe response surface design.[4]When one needs to optimize more than one response at a time, the use of multicriteria decision making (MCDM), a chemometric technique, is the best choice. Chemometrics can be used to accomplish a variety of goals in chromatography laboratory: (i)

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speeding methods development, (ii) makingbetter use of chromatographic data, and (iii) explaining the chromatographic process.[5]

In this response surface methodology (RSM) technique, the main objective is to optimize the response surface that is influenced by various process parameters. Response surface methodology also quantifies the relationship between the controllable input parameters and the obtained response surfaces.[6]Mostapplications of RSM are sequential in nature and can be carried out based inthe following phases:

Phase0**:** Screening experiment; identification of the independent variables

Phase 1**:** Independent variables result in a value that is near the optimum

Phase 2**:** Model may be analyzed to find the optimum conditions for the process.

The desirability function approach is one of the most frequently used multi-response optimization techniques in practice. There are many ways in which the individual desirabilities can be combined. If the combined criterion is a simple arithmetic average, it is called a utility function, and if it is a geometric mean, it is referred to as Derringer's desirability function. The idea of combining desirabilitiesas a geometric mean was first presented by Harrington,[7] but it was put into a more general form by Derringer.[8]The advantage of the Derringer's desirability function is that if one of the criteria has an unacceptable value, then the overall product will also be unacceptable, while for the utility functions, this is not the case. Further, the Derringer's method offers the user flexibility in the definition of desirability functions. Derringer's desirability function was introduced in chromatography by Deming,[9]implementing resolution and analysis time as objective functions to improve separation quality. The desirability lies between 0 and 1, and it represents the closeness of a response to its ideal value. If a response falls within the unacceptable intervals, the desirability is 0, and if a response falls within the ideal intervals or the response reaches its ideal value, the desirability is 1.The more closely the response approaches the ideal intervals or ideal values, the closer the desirability is to 1.Lamivudine (LMI), chemically known as (2R,cis)-4-amino-1-(2-(hydroxylmethyl-1,3-oxathiolan-5-yl)-(1H) pyrimidin-2-one, is a nucleoside-reverse transcriptase inhibitor (NRTI). It is an analogue of cytidine. Tenofovir disoproxil fumarate (TDF), chemically known as 9-((R)-2-((bis (((isopropoxycarbonyl) oxy) methoxy) phosphine) methoxy) propyl) adenine fumarate (1:1), is a nucleotide analogue reverse transcriptase inhibitor (nRTIs).Efavirenz (EFV), chemically known as (4S)-6-chloro-4-(cyclopropylethynyl)4-(trifluromethyl)-1-4-dihydro-2H-3,1-benzoxazin-2-one, is an antiretroviral drug which is a non-nucleoside reverse transcriptase inhibitor (NNRTI).[10-11]The estimation of lamivudine using UV spectroscopy and HPLC has been reported. The estimation of TDF by RP-HPLC has been reported. TDF has been determined in spiked human plasma by HPLC. EFV has been determined by UV spectroscopic and RP-HPLC methods in single and in combined dosage forms.[12]Although the combination of EFV, TDF, and LMI is not available commercially in the market, it is in phase 3 clinical trial and the safety and efficacy of TDF in combination with LMI and FFV has already been reported.

This study revealed that a once daily regimen containing EFV, TDF, and LMI is virologically and immunologically effective, well tolerated and safe with benefits in the lipid profile in the majority of patients. Literature survey revealed that only one spectroscopic method has been reported for 3 drugs in combination and no chemometric method developed for optimization of chromatographic system. Hence, an attempt was made for chemometric optimization for simultaneous determination of lamivudine, tenofovir,and efavirenz by RP-HPLC as per ICH guidelines. These drugs are not official in any of the pharmacopoeias. These are listed in the Merck Index and Martindale.The complete drug references are shown in figure1.

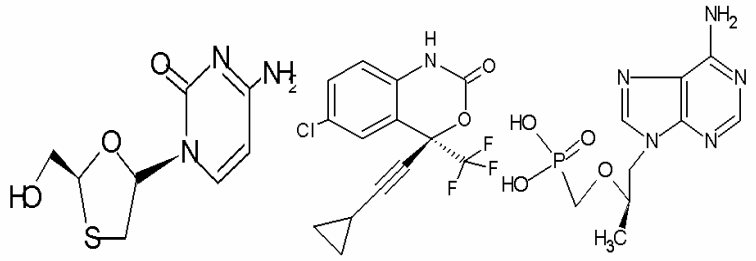

Figure 1.The chemical structures of LMI, TDF, and EFV

EXPERIMENTAL

Apparatus

Shimadzu prominence High Performance Liquid Chromatography with PDA detector and auto-injector mode was used with LC Empower software. The system was controlled through a system controller and a personal computer using Shimadzu chromatographic software installed on it. Absorbance spectra were recorded using an UV–visible spectrophotometer employing a quartz cell of 1.00 cm of path length.

Software

Experimental design, data analysis and desirability function calculations were performed by using Design-Expert®, trial version 7.0. (Stat-Ease Inc., Minneapolis).

Chemicals and Reagents

Working standards of lamivudine (99.79%), tenofovir (99.78%), and efavirenz (99.76%) were donated by M/S Pharma Train, Hyderabad, India. Methanol (MeOH) HPLC grade andtriethylamine analytical-reagent grade were supplied by M/S SD Fine Chemicals, Mumbai, India. The HPLC grade water was prepared by using a Milli-Q Academic, Millipore, Bangalore, India.

Standard Solutions

Seventy fivemg of lamivudine, 75 mg of tenofovir, and about 150 mg of efavirenz working standards were weighed accurately and transferred into a 50 mL clean and dry volumetric flask.TenmL of diluent was added and sonicated to dissolve. Then it was diluted to volume with diluent and mixed.

Working Standard Solution

Five mL of the above solution was diluted to 50 mL with diluent and mixed well. Then it was filtered through a 0.45µ membrane filter, so the final concentrations were 150 µg/mL of lamivudine, 150 µg/mL of tenofovir,and about 300 µg/mL of efavirenz.

Sample Preparation

Ten tablets were accurately weighed and crushed into a fine powder. The powder equivalent to one tablet (300 mg of lamivudine, 300 mg of tenofovir disoproxil fumerate, and 600 mg of efavirenz) was taken in 500 ml volumetric flask. About 250 ml diluent was added, shaken for 5 min on a rotary shaker and then sonicated for 20 minutes with intermediate shaking. After that, the volume was finally made up to the mark (500ml). A sample solution was centrifuged at 5000 rpm for 5 minutes to get a clear solution. Then 5 ml supernatant solution was diluted to 100 ml with diluent.It was filtered through a 0.45µ membrane filter, so the final concentrations were 150 µg/mL of lamivudine, 150 µg/mL of tenofovir, and about 300 µg/mL of efavirenz.

Chromatographic Procedure

Chromatographic separations were carried out on a Hyper Thermosil Column C_{18}(4.6 x 150mm, 5 µm, Make: ACE). The mobile phase consisted of a methanol–triethylamine buffer (pH 3.0), adjusted with 10% phosphoric acid. In order to increase the sensitivity for the less concentrated compound and to decrease the background from the mobile phase, a wavelength of 260 nm was selected for detection. An injection volume of the sample was 20 µL. The HPLC system was used in an air conditioned laboratory atmosphere (20±2 ◦C).

RESULTS AND DISCUSSION

Optimization Design and Analysis

In this work, the important chromatographic factors were selected and optimized by a central composite

design experiment. The selection of factors for optimization was based on preliminary experiments and prior knowledge from literature, as well as certain instrumental limitations. For instance, the mobile phase pH was fixed at 3.0 as this could influence the stability.[13]From preliminary experiments,the mobile phase consistingof a methanol and triethylamine buffer was employed in which the concentration of methanol content was varied.[14]The mobile phase flow rate could also moderately influence selectivity in the HPLC analysis. Therefore, the key factors selected for the optimization process were methanol concentration (*A*), buffer molarity (*B*), and flow rate (*C*). Table 1 shows the levels of each factors studied for finding out the optimum values and responses. As can be seen in this table, the ranges of each factor used were: methanol concentration (60–70%), buffer molarity (15–25 mM), and flow rate (0.7–0.9 ml/min).

Table1:Central composite rotatable design arrangement and responses

Design points	**Factor levels**			**Responses**		
Run order	**A (mM)**	**B (%v/v)**	**C (mL/min)**	**Capacity factor**	**Resolution**	**Retention time**
1	20	65	0.8	1.51	11.44	11.23
2	20	65	0.97	1.37	10.63	10.43
3	15	70	0.9	1.32	9.45	9.49
4	20	65	0.8	1.49	10.67	10.5
5	25	60	0.9	1.55	12.76	12.4
6	20	73.41	0.8	1.45	9.36	8.32
7	25	70	0.9	1.5	8.86	8.45
8	28.41	65	0.8	1.73	11.44	9.43
9	20	56.59	0.8	1.45	16.87	17.6
10	25	60	0.7	1.58	11.32	13.2
11	20	65	0.8	1.48	10.65	10.47
12	20	65	0.8	1.49	10.78	10.45
13	15	60	0.9	1.35	16.72	15.5
14	20	65	0.63	1.57	10.67	10.21
15	11.59	65	0.8	1.25	10.78	11.63
16	25	70	0.7	1.55	8.15	8.41
17	20	65	0.8	1.49	10.78	10.45
18	15	70	0.7	1.39	8.86	9.52
19	20	65	0.8	1.48	10.72	10.45
20	15	60	0.7	1.37	16.56	15.2

As response variables, the capacity factor of lamivudine (k_1), the resolution between tenofovir and efavirenz ($Rs_{2,3}$), and the retention time of efavirenz (tR_3) were chosen. All experiments were performed in randomized order to minimize the effects of uncontrolled variables that may introduce a bias on the measurements. For an experimental design with three factors, the model including linear, quadratic, and cross terms can be expressed as:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_{21} + \_\beta_{22} X_{22} + \beta_{33} X_2$$

where Y is the response to be modeled, β is the regression coefficient, and $X1$, $X2$ and $X3$ represent factors A, B and C, respectively. To obtain a simple and yet realistic model, the insignificant terms ($P > 0.05$) are eliminated from the model through a 'backward elimination' process. The statistical parameters obtained from the ANOVA for the reduced models are given in table 2.Since R^2 always decreases when a regressor variable is eliminated from a regression model, in statistical modeling the adjusted R^2, which takes the number of regressor variables into account, is usually selected. Adjusted $R^2$was defined as: 1- SS_E (n-p) / SS_T (n-1) = 1-(n-1)/(n-p)x(1-R^2)

Table2: Reduced response surfaces models and statistical parameters obtained from ANOVA

Response	Regression model	Adjusted R^2	Model P-value	%C.V	Adequate precision
k_1	+1.47+0.11* A-0.037* C	0.885	0.0001	2.48	27.215
Rs_{23}	+10.84-0.69* A-2.54* B+0.99*A* B+0.79* B^2	0.8898	0.0001	7.39	20.327
tR_3	+10.56-0.80*A-2.64* B+0.37 *A*B+0.88* B^2	0.9847	0.0001	2.76	59.277

In the present study, the adjusted R^2 was well within the acceptable limits of $R^2 \geq 0.80$ [15] which revealed that the experimental data shows a good fit with the second-order polynomial equations. For all the reduced models, P value of <0.05 wasobtained, implying these models are significant.The adequate precision value is a measure of the "signal (response) to noise (deviation) ratio". A ratio greater than 4 is desirable. In this study, the ratio was found to be in the range of 20.327-59.277, which indicates an adequate signal and, therefore, the model is significant for the separation process. The coefficient of variation is a measure of reproducibility of the model and as a general rule a model can be considered reasonably reproducible if it is less than 10%. The coefficient of variation for all the models was found to less than 10%. The results are shown in table2. Hence, the diagnostic plots, (a) the normal probability plot of the residuals and (b) the plot of the residuals versus predicted values, were analyzed for response $Rs_{2,3}$. Since the assumptions of normality and constant variance of the residuals were found to be satisfied, the fitted model for the $Rs_{2,3}$was accepted. In table 2, the interaction term with the largest absolute coefficients among the fitted models is AB (+0.37) of the tR_3 model. The study reveals that changing the fraction of MeOH from low to high results in a rapid decline in the retention time at the low and high levels of buffer molarity. Further at the low level of factor B, an increase in the buffer molarity results in a marginal decrease in the retention time. Therefore, when the MeOH concentration is set at its lowest level, the buffer concentration has to be at its highest level to shorten the run time. This interaction is synergistic as it led to a decrease in run time. In order to gain a better understanding of the results, the predicted models are presented in figure 2 as the perturbation plot. For an optimization design, this graph shows how the response changes as each factor moves from a chosen reference point, with all other factors held constant at the reference value. A steep slope or curvature in a factor indicates that the response is sensitive to that factor. Hence, the plot shows that factor B mostly affected the analysis time tR_3 followed by factor A and then C.

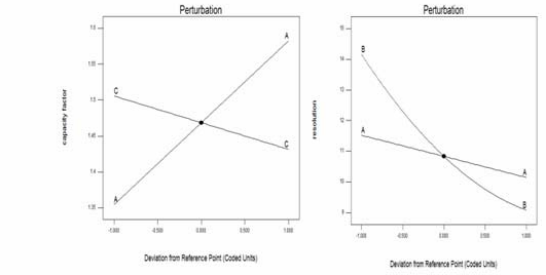

Figure2.A

Figure2.B

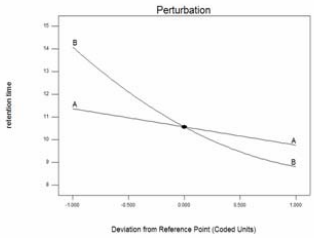

Figure2.C

Figure 2. Perturbation plots for responses
2.A:Perturbation plot for capacity factor response
2.B: Perturbation plot for resolution response
2.C: Perturbation plot for retention time response

Multi-criteria Decision Making

In the present study, to optimize the three responses with different targets, Derringer's desirability function, was used.[8]The Derringer's desirability function(*D*) is defined as the geometric mean, weight, or otherwise, of the individual desirability functions. Desirability function di = Di(Yi)for each response separately (igoes from 1to the number response say q) The expression that defines the Derringer's desirability function is:

$D = [d_1^{p1} \times d_2^{p2} \times d_3^{p3} \times \cdots \times d_n^{pn}]^{1/n}$

where Pi is the weight of the response, n the number of responses and di is the individual desirability function of each response obtained from the transformation of the individual response of each experiment. The scale of the individual desirability function ranges between $d^i = 0$, for a completely undesired response, to $d^i = 1$ for a fully desired response.Weights can range from 0.1 to 10. Weights lower than 1 give less emphasis to the goal whereas weights greater than 1 give more emphasis to the goal. A value of D different thanzero implies that all responses are in a desirable range simultaneously.Consequently, for a value of D close to 1, the combination of the different criteria is globally optimal, so the response values are near the target values. The criterion for the optimization of each individual response isshown in table 3. The criteria were proposed for selecting an optimum experimental condition for analyzing routine quality control samples. As can be seen under the criteria, two responses tR_3 and $Rs_{2,3}$ were minimized, in order to shorten the analysis time.

Table 3:Criteria for the optimization of the individual responses

Response	Lower limit	Upper limit	Criteria/Goal
k_1	1.18	1.78	Target =1.5
$Rs_{2,3}$	7.76	12.3	Minimize
tR_3	8.92	17.6	Minimize

Table 4: Comparison of experimental and predictive values of different functions under optimal conditions

Optimum conditions	**Buffer (mM)**	**Methanol (%v/v)**	**Flow rate (mL/min)**	**k_1**	**$Rs_{2,3}$**	**tR_3**
Predictive	23	70	0.9	1.5	9.26	8.54
Experimental	23	70	0.9	1.48	9.15	8.32
Average error				1.33	1.18	2.57
Desirability value (D) =0.95						

In order to separate the first eluting peak lamivudine from the solvent front, k_1 was targeted at 1.5. Importance can range from 1 (the least important) to 5 (the most important), which gives emphasis to a target value. For instance, a high importance value of 4was assigned to the tR_3 response as a short analysis time is usually preferred for routine analysis. Following the conditions and restrictions above, the optimization procedure was carried out. The response surface obtained for the global desirability function is presented in figure3. The coordinates producing the maximum desirability value (D= 0.95) were a methanol concentration of 70%, buffer molarity of 23mM and flow rate of 0.9 ml/min. The predicted response values corresponding to the latter value of *D* werek_1 = 1.5, $Rs_{2,3}$ = 9.26, and tR_3 = 8.54 min. The prediction efficiency of the model was confirmed by performing the experiment under the optimal condition and the corresponding chromatogram is shown in figure4.

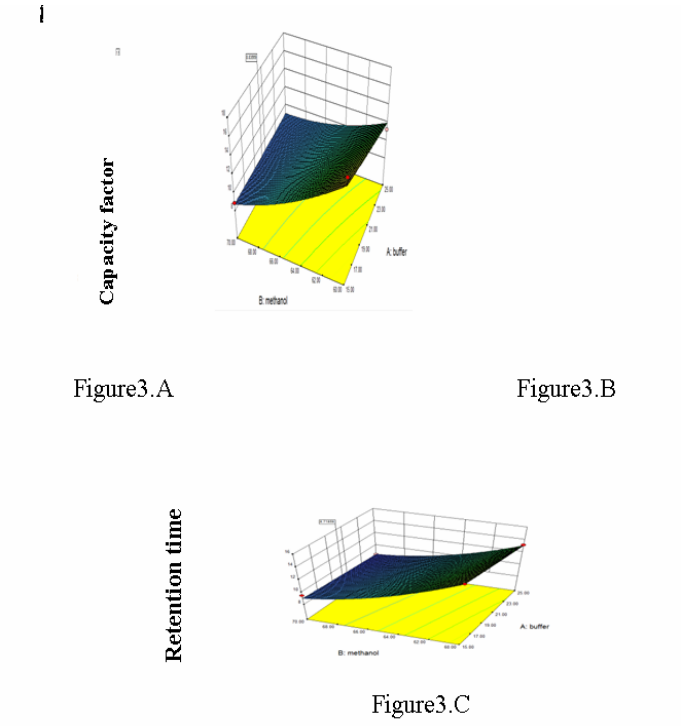

Figure3.A Figure3.B

Figure3.C

Figure 3. Desirability plots for responses

Graphical representation for capacity factor response. Buffer concentration (A) is plotted against methanol (B)
Graphical representation of resolution factor. Buffer concentration (A) is plotted against methanol (B)
Graphical representation of retention time response. Buffer concentration (A) is plotted against methanol (B)

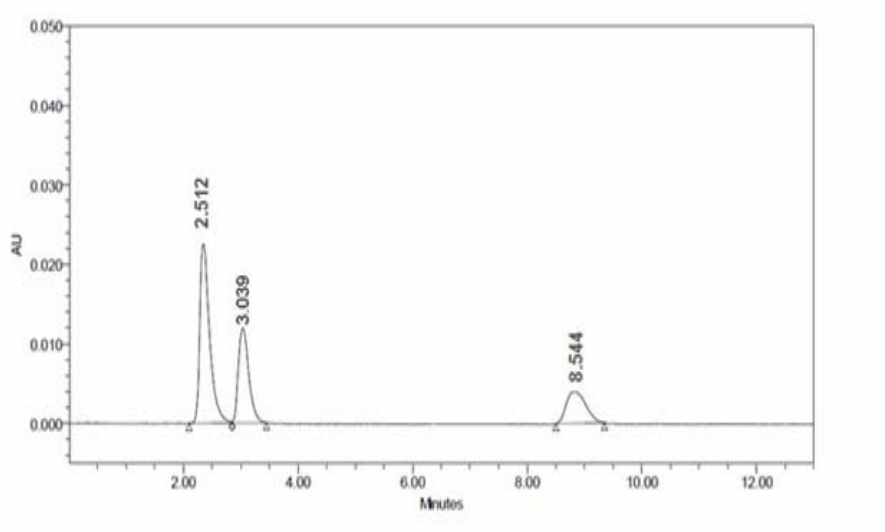

Figure 4.Chromatogram for optimized assay method

Assay Method Validation

The optimized assay method is specific in relation to the placebo used in this study because there was no excipient peak co-eluted with the analytes (figure 4). An excellent linearity was established at five levels in the range of 75-225µg/ml for lamivudine, tenofovir,and 150–

450µg/ml for efavirenz, with R^2 of more than 0.999. The LOD were estimated as 5.185, 5.038, and 8.731µg/ml and LOQ were estimated as 15.713, 15.268, and 26.462µg/ml, respectively forlamivudine, tenofovirand efavirenz. Accuracy assessed by spike recoverywere found to be 100.28, 99.43 and 99.91%, respectively, which were within acceptable ranges of 100±2%. The intra- and inter-assay precision (n = 6) was confirmed since the %RSD was found to be less than 2. The robustness study reveals that small changes did not alter the retention times, capacity factor, and resolutions more than 4%, and therefore, it would be concluded that the method conditions are robust.

Conclusion

The analytes lamivudine, tenofovir, and efavirenz have been simultaneously analyzed in pharmaceutical formulations by using HPLC. Time of analysis, resolution, and quality of the peaks were simultaneously optimized by applying useful tools of chemometrics: response surface design and Derringer's desirability function. The validation study supported the selection of the assay conditions by confirming that the assay was specific, accurate, linear, precise, and robust. The results of the study demonstrate the benefit of applying this approach in selecting optimum conditions for the determination of drugs in pharmaceutical formulations. This method reduces overall assay development time and provides essential information regarding the sensitivity of various chromatographic variables on separation attributes.

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التحليل الكيميائي الأمثل في الوقت نفسه لتعيين الأدوية المضادة لفيروس القهقرينتيجة للطورالعكسي- انفصال السائل ذو الفعالية العالية نظريا

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ملخـص

لصنف الطلب في هذا العمل، وتحقيق الأمثلية وفي الوقت نفسه نستخدم رغبة وظيفة درينجير لتنمية الطورالعكسي- انفصال السائل ذي الفعالية العالية وفي الوقت نفسه لتحديد وتعيين الأدوية مثل لاميفودين، تينوفوفير، إيفافيرينز في تحضير منتجات الأدوية التجارية. عشرون تجربة تأخذ بعامل السعة كأول عامل قمة،والقرار بين القمة الثانية والثالثة والاحتفاظ بالزمن الثالث كمسؤول عن ثلاث متغيرات مهمة مثل طور التكوين العضوي، العازلة المولية ومعدل التدفق الذي يستخدم لتصميم النماذج الحسابية. ردود الفعل التجريبية مصنوعة حسب المقاس إلى الترتيب الثاني (بولينوميال) والرد الثالث في آن واحد ليتنبأ أفضل حالة للتأثير الانفصالي للمركبات المدروسة. أفضل حالة للتعيين هي ميثانول- ثلاثي إيثيل أمين كعازل (الرقم الهيدروجيني 3.0- 15.5 مم) (35:65 % v/v/v) كطور متحرك بمعدل تدفق 1.19 مل \ دقيقة، بينما يستخدم أفضل حالة، الأساس هو الانفصال مع الحد الأدنى 2 وتحقيق الوقت في أقل من 6 دقائق. هذه الطريقة تظهر أفضل اتفاق بين معطيات التجربة والرقم التخميني من خلال العامل المحدد المدروس. أفضل تعيين هو على حسب المؤتمر التنسيقي الدولي الذي يؤكد بالرسم تحديدا والضبط والدقة.

الكلمـات الدالـة: تعدد رد الفعل وتحقيق الأمثلية: رغبة وظيفة درينجير :للطور العكسي- انفصال السائل ذي الفعالية العالية لتصميم المركب الرئيس مثل لاميفودين، تينوفوفير دايسوبروكسيل فيوميرات وإيفافيرينز.

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