Using Design Space and Response Surface Methodology for developing a liquid chromatography method for simultaneous determination of five statins in pharmaceutical form

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ABSTRACT

This study describes the development of a method allowing the simultaneous separation and quantification of five statins by High performance liquid chromatography/Diode Array Detector (HPLC/DAD). Optimization was accomplished using chemometric tools such as the Design Space (DS) and Response Surface Methodology (RSM). Central Composite Design (CCD) and DS were applied for the optimization of the chromatographic procedure as well as the robustness of the chromatographic method by taking the ratio of the percentage of acetonitrile (%ACN) Buffer solution, the pH and the mobile phase flow rate as critical parameters. Satisfactory results were obtained after the optimization phase with a percentage of mobile phase equal to 46.19%, a pH of 4.16 and the flow rate is 1.4 mL min⁻¹ by setting the resolution limits above 6, and the target retention time of 20 min. Using the DS and CCD approach, we have developed a robust and reliable procedure for the simultaneous and accurate separation and quantification of the five statins.

KEYWORDS

statins, HPLC/DAD, Design Space, Central Composite Design, Plackett-Burman Design

INTRODUCTION

Statins are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors. It is a new class of drug more prescribed for the treatment of hypercholesterolemia in patients with cardiovascular disease and those at high risk of developing atherosclerosis [1]. It was from the 1970s onwards that statins underwent a very important development in the pharmaceutical field due to their lipid-lowering properties, which present a main mechanism for preventing the development of atherosclerosis [2]. Several statins are available on the market for the treatment of hypercholesterolemia: Lovastatin marketed in (September 1987), Simvastatin (1988), Pravastatin in (October 1991), Fluvastatin in (April 1994), Atorvastatin in (1997), Cerivastatin (1988), and Rosuvastatin (2003). Any compound that shares this pharmacological trait, regardless of its chemical structure, is likely to be suffixed "statin". The development of new statins has always been the concern of the pharmaceutical industry, but their analysis presents a rigorous problem for analysts. Our study is looking at five statins, namely Lovastin, Simvastin, Pravastatin, Atorvastatin and Rosuvastatin, which are the best-known statins in the anti-hypercholesterolemia drug market. Their structures are summarized in Fig. 1, which are translated into different physicochemical properties.
In addition, a literature review revealed that there is no High performance liquid chromatography/Diode Array Detector (HPLC/DAD) method available for simultaneous estimation of all five statins in one pharmaceutical form, and that the Quality by Design (QbD) approach has not been used for the development of the HPLC/DAD separation of the five statins. However, a few analytical methods have been reported in the literature for the determination of each form of statin alone or in combination with other drugs, including spectrophotometric methods for the determination of statins in pharmaceutical form and other methods such as HPLC with UV detection [3]. In addition, regulatory authorities (FDA, ICH) encourage and recommend the application of the QbD approach to understand chromatographic selectivity and promote better control of methods including the transfer method. This has prompted researchers to adopt the QbD approach to the development of HPLC methods, and many papers have been published concerning this use [4–9]. QbD is a systematic approach that includes multidimensional combinations of input variables using design of experiments such as Response Surface Methodology (RSM) to obtain optimal conditions with better quality assurance [10]. Design Space (DS) is a key step in the QbD approach. It is used to establish a multidimensional space based on the relationship between the measured responses and the critical parameters of the method, this relationship is exploited and estimated by using the RSM [11]. In addition, RSM is a tool used to gain maximum understanding of the effects and interactions between the most critical process parameters in order to provide an optimal and robust analytical method. Several key steps were involved in study. Firstly, before the application of the RSM, the critical parameters affecting the method must first be selected by the screening study. Since the sample size is traditionally small, the interaction effects are completely enveloped in the main effects. Therefore, the Plackett–Burman Design (PBD) only determines which of several experimental variables has more significant effects [12–21]. Secondly, the selected parameters are optimized by using the RSM. Among the best-known response surface methodologies are Box-Behnken Design (BBD), Central Composite Design (CCD), and Doehlert Design (DD). In this work, we opted for the CCD method [22]. Indeed, the CCD was presented by Box and Wilson [23], it is a sequential design, because it consists of three parts: Factor design, star points and center points [24]. Factorial designs are two-level designs (−1 and +1), which allow the interactions to be studied and the model to be determined. The star points (or axial points), these points are located on the axes of each of the factors. The center points are extremely useful because they allow to test the validity of the first-degree model, to certify the stability of the model, to have an estimate of the experimental error, to decrease the prediction error near the central point [24]. Then, the DS is constructed and used to show the flexible region allowing to scientifically evaluate the impact of any deliberate change in the method’s knowledge space [10]. In order to have a better resolution and a shorter analysis time, we used the same methodology for the development and optimization of a new HPLC/DAD method allowing the separation of the five statins in order to quantify them simultaneously and accurately. Our work consists of identifying failure modes (Critical Factors) and establishing a robust DS. Here, the desired state of robust DS is based on systematic multivariate experiments. For this we proceeded by a multivariate optimization using the RSM/CCD which allowed us to build the DS.

**EXPERIMENTAL**

**Reagents and chemicals**

The working standards were provided by the Drug Control Laboratory (NCML), Acetonitrile, sodium acetate, and methanol (HPLC grade) were obtained from Sigma-Aldrich (Germany). The placebo consists of a mixture of the following excipients: (crospovidone E1202), titanium dioxide TiO₂, lactose C₁₂H₂₂O₁₁, iron oxide yellow, and red E172, corn starch, pregelatinized, magnesium stearate E572, citric acid E330, microcrystalline cellulose E460, methyl propyl cellulose, hydroxypropyl cellulose e463, opadryl white, BHA (butylated hydroxyanisole) E320, povidone k30.

**Chromatographic equipment and conditions**

Chromatographic analysis was performed in this study with a fully automated system named (WATERS 2695) equipped with the following components: A Model 2695 quaternary pump, a thermostatically controlled automatic injector, a thermostatically controlled column station and a Model 2998 iodine strip UV detector. The data were acquired and processed with a data logging software program (Empower Software). The separation was carried out with a flow rate of 1.3 mL min⁻¹ using a Waterspherisorb ODS1 C18 RP type column (4 × 250 mm; 5 μm). The mobile phase is composed of a mixture of sodium acetate buffer solution
adjusted to a value of (pH = 3.8) (45:55; v/v). The buffer solution of pH = 3.8 was prepared by dissolving 1 g of sodium acetate in 1 L of water, the pH was adjusted to 3.8 with acetic acid. The column was maintained at 30 °C. The UV detection wavelength is 238 nm. The pH of the buffer solution was measured with a pH meter (Prolab 300).

Preparation of the solution for the development study

A concentration of 0.1 mg mL\(^{-1}\) of Lovastatin, Simvastatin, Pravastatin, Atorvastatin, and Rosuvastatin, in a mixture of acetonitrile and water (50%-50%), was prepared for screening and optimization tests.

Procedure

**Plackett–Burman Design (PBD).** The screening of process parameters for chromatographic separation was carried out with a PBD. The PBD is a fraction of a two-level factorial design that allows to examine the N-1 variables with at least N experiments. It is principally used to select and evaluate important factors that appear to influence the selected responses in this study, namely the Rt of the last peak, the Pravastatin and Rosuvastatin (RPR) and the Rosuvastatin (RRA). The factors selected for the screening study are grouped in Table 1. Five factors selected in this study were tested at two levels according to the PBD experimental matrix shown in Table 2.

The PBD is essentially based on a first-order model that does not describe any interaction between the factors:

\[
Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \varepsilon
\]  

where \(Y\) is the experimental response and \(X_1, X_2, X_3, X_4, \) and \(X_5\) are coded variables corresponding to ammonium acetate concentration, percentage of acetonitrile (%ACN), column temperature, pH, and flow rate, respectively. The coefficients \(\beta_1, \beta_2, \beta_3, \beta_4, \text{ and } \beta_5\) are the main effects of each variable studied\(^{11}\). The screening study will allow to determine the weight of each level for each factor on the selected responses, and then rank them in order of importance according to the "Pareto" principle.

**Central Composite Design (CCD).** The objective of the PBD was to study all the factors in order to select the most significant ones. The CCD, on the other hand, aims to seek the optimal values that the selected factors can take to achieve our objectives. The objectives are explained by responses such as reduced Rt and good resolutions between peaks. The CCD has 3 levels, the first level is a factorial design consisting of 8 trials which are located at the vertices of the cube, the second level consists of 6 star trials (6 points located on the axes of the cube all at the same distance from the origin equal to \(\alpha = 1.682\)). As for the third level, it consists of 6 replications carried out at the center, leading finally to 20 experiments. Based on the results of the screening study, the %ACN \((X_1)\), the pH of the buffer solution \((X_2)\) and the mobile phase flow rate \((X_3)\) were identified as influential factors and were selected for the method optimization study. The three factors evaluated in this Design and their levels are listed in Table 3. The optimization by the CCD, with 3 factors and 3 levels, was carried out using the MINITAB software in order to obtain RPR and RRA greater than or equal to 6 and a target Rt of 20 min.

The experimental designs of the actual levels of the variables are presented in Table 4. Responses \(Y_1, Y_2, \text{ and } Y_3\) were related to the coded variables \(X_6, i = 1, 2, 3\) by a

### Table 1. Factors and levels studied for screening using a PBD

| Independent factors          | Unit | Symbol | Low | High |
|------------------------------|------|--------|-----|------|
| Concentration of ammonium acetate | M    | \(X_1\) | 0.8 | 1.2  |
| Percentage of acetonitrile | %    | \(X_2\) | 45  | 55   |
| Column temperature           | °C   | \(X_3\) | 25  | 35   |
| pH                           | -    | \(X_4\) | 4   | 4.6  |
| Flow rate                    | mL   | \(X_5\) | 1.1 | 1.5  |

### Table 2. The PBD experimental matrix with 5 variables

| Run | Conc. | %ACN | \(T^\circ\) | pH  | Flow rate | Rt   | RPR | RRA |
|-----|-------|------|------------|-----|-----------|------|-----|-----|
| 1   | 1.2   | 45   | 35         | 4.0 | 1.1       | 30.87| 7.43| 13.66|
| 2   | 1.2   | 55   | 25         | 4.6 | 1.1       | 3.02 | 1.44| 1.53|
| 3   | 0.8   | 55   | 25         | 4.0 | 1.5       | 8.56 | 2.48| 4.25|
| 4   | 1.2   | 45   | 35         | 4.6 | 1.1       | 31.44| 2.78| 4.35|
| 5   | 1.2   | 55   | 25         | 4.6 | 1.5       | 9.41 | 2.46| 3.59|
| 6   | 1.2   | 55   | 35         | 4.0 | 1.5       | 8.84 | 4.24| 6.98|
| 7   | 0.8   | 55   | 35         | 4.6 | 1.1       | 12.32| 1.82| 2.51|
| 8   | 0.8   | 45   | 35         | 4.6 | 1.5       | 12.93| 1.81| 3.16|
| 9   | 0.8   | 45   | 25         | 4.6 | 1.5       | 25.68| 2.45| 3.49|
| 10  | 1.2   | 45   | 25         | 4.0 | 1.5       | 23.84| 5.08| 10.10|
| 11  | 0.8   | 55   | 25         | 4.0 | 1.1       | 12.59| 2.74| 4.19|
| 12  | 0.8   | 45   | 25         | 4.0 | 1.1       | 33.77| 6.25| 9.62|

Conc: Concentration of ammonium acetate; %ACN: Percentage of acetonitrile; \(T^\circ\): Column temperature.
RESULTS AND DISCUSSIONS

Preliminary study

Before beginning the screening study, a univariate preliminary study is important in order to select the essential factors for a successful study. The choice of the column always remains an essential step for an HPLC method. Day after day the competition in the column industry doesn’t stop, and the analyst was faced with a multitude of columns. The problem lies in the fact that columns coated with silica gel remarkably grafted by C18 hydrocarbon chains with the same characteristics (column length and particle diameter) give different results. This difference may be due to other parameters that characterize one column to another, such as the pore size of the particles, the surface area, the carbon content, and the number of theoretical plateaus. We have tested a very large number of columns in order to choose the one that gives good results (a good resolution and a suitable retention time). A high-performance column from Waters Spherisorb ODS1 was chosen due to the satisfactory results obtained. The other factors that characterize a chromatographic method will be studied during the screening step. An initial test of a sample containing the five statins led us to obtain the chromatogram in Fig. 2.

The chromatogram in Fig. 2 shows that the retention time for the three statins is 2.5, 4.9, and 14.5 min for Pravastatin, Rosuvastatin, and Atorvastatin respectively. For Lovastatin and Simvastatin, you must wait more than 60 min. The goal now is to identify the five statins in a timely manner. However, to reduce the retention time (Rt) of Lovastatin and Simvastatin, it is possible that even the Rt of Pravastatin, Rosuvastatin and Atorvastatin will be reduced, and consequently a poor resolution will be generated between RPR and between RRA.

Screening study by PBD

Based on the results of the Plackett–Burman screening study translated into Pareto diagrams of the three responses in Figs. 3–5, it is clear that the %ACN and pH are the most influential factors on Rt, RPR, and RRA. While the flow rate has more influence on Rt than on RPR and RRA, and the concentration of ammonium acetate has more influence on RPR and RRA than on Rt. However, from a practical point of view, the flow rate is a very important factor in HPLC analysis and separation since it has a lot of influence on the system pressure, the flow of the mobile phase in the column and it also plays a very important role in the Rt of the compounds. For this reason, we selected the flow rate of the mobile phase instead of the concentration of ammonium acetate next to the %ACN and the pH of the buffer as factors subject to optimization to improve RPR and RRA, and reduce the Rt of the five statin assay method.

Optimization study by CCD

Statistical analysis. The analysis of variance is a statistical test that verifies the validity of the model applied for before
beginning the optimization study using the response surfaces. Even if a $P$-value is found to be less than 0.05 indicating the significance of the test, in our case we only consider a result to be significant and the model accepted only if the $P$-value is less than 0.01. Tables 5–7 summarize the results of the analysis of variance for the three models (for all three responses), show that all factors have a significant main effect ($P$-value <0.01), except for the pH factor in the model for the Rt response (Table 5) and the flow rate factor for the RPR (Table 6) and RRA responses (Table 7). Regarding the
The analysis of variance showed that the model represents the phenomenon well and that the variation of responses was correctly related to the variation of factors, i.e., the regression variation is greater than the variation of residuals. Therefore, the models are considered valid and their equations considered are as follows:

\[
y_1 = 24.95 - 8.94X_1 + 6.36X_3 + 7.039X_1^2 + 1.57X_3^2 + 3.53X_1X_3
\]

\[
y_2 = 6.183 - 2.041X_1 - 1.248X_2 + 0.170X_1X_2
\]

\[
y_3 = 11.096 - 4.976X_1 - 2.360X_2 + 0.755X_1^2 + 1.473X_1X_2
\]

**Response-surface plots.** In order to graphically present the three-dimensional relationship between the factors and the different responses studied in a 2D and 3D format, a contour plot and response surface plots have been drawn. The contour plot for Rt illustrated in Fig. 6 shows that Rt values vary differently with increasing pH and percentage acetonitrile, in fact the Rt decreases as the %ACN increases. On the other hand, only a minimal influence of pH was observed. However, Fig. 7, shows the response surface plots for the two resolutions RPR and RRA, and demonstrates that the resolution evolves inversely with pH, as an increase in pH leads to a slight decrease in resolution, with regard to the percentage of the mobile phase (%ACN), an increase in this latter causes a sharp decrease in resolution. Thus, the area of highest resolution is observed at the ratio of the lowest %ACN value to the lowest pH value.

**Design Space optimization.** The DS was established to minimize Rt and maximize RPR and RRA based on predictive statistical models of these three responses. The construction of DS requires a prior determination of the potential conditions, namely a good RPR and RRA which should be greater than or equal to 6 and a Rt around 20 min. The white area of the DS in Fig. 8 represents the description of the multidimensional interaction effects, it was noted from the ANOVA table that only the effects of %ACN × pH for the Rt response (Table 5) and %ACN × pH for the RPR (Table 6) and RRA responses (Table 7) were found to be significant. As for the quadratic terms, except for the quadratic effects of %ACN for Rt response (Table 5) and RRA responses (Table 7) and the flow rate on Rt response (Table 5), the others are all insignificant.

**Table 6. Regression coefficients and their significance in the quadratic model of RPR response and the ANOVA of response surface quadratic model.**

| Source        | DF | SS       | MS         | F-value | P-value |
|---------------|----|----------|------------|---------|---------|
| Model         | 9  | 88.4561  | 9.8285     | 14.04   | 0.0001  |
| Linear        | 3  | 78.2948  | 26.0983    | 37.29   | <0.001  |
| %ACN          | 1  | 56.8930  | 56.8930    | 81.29   | <0.001  |
| pH            | 1  | 21.2554  | 21.2554    | 30.37   | 0.0003  |
| Flow rate     | 1  | 0.1464   | 0.1464     | 0.21    | 0.657   |
| Square        | 3  | 1.8983   | 0.6328     | 0.90    | 0.473   |
| %ACN × %ACN   | 1  | 0.4175   | 0.4175     | 0.60    | 0.458   |
| pH × Flow rate| 1  | 0.0900   | 0.0900     | 0.13    | 0.727   |
| Flow rate ×   | 1  | 1.2517   | 1.2517     | 1.79    | 0.211   |

**Table 7. Regression coefficients and their significance in the quadratic model of RRA response and the ANOVA of response surface quadratic model.**

| Source        | DF | SS       | MS         | F-value | P-value |
|---------------|----|----------|------------|---------|---------|
| Model         | 9  | 449.180  | 49.909     | 44.87   | <0.001  |
| Linear        | 3  | 416.346  | 138.782    | 124.76  | <0.001  |
| %ACN          | 1  | 338.150  | 338.150    | 303.99  | <0.001  |
| pH            | 1  | 76.049   | 76.049     | 68.38   | <0.001  |
| Flow rate     | 1  | 2.147    | 2.147      | 1.93    | 0.195   |
| Square        | 3  | 13.525   | 4.508      | 4.05    | 0.040   |
| %ACN × %ACN   | 1  | 8.220    | 8.220      | 7.39    | 0.022   |
| pH × Flow rate| 1  | 0.150    | 0.150      | 0.14    | 0.721   |
| Flow rate ×   | 1  | 3.834    | 3.834      | 3.45    | 0.093   |

**Fig. 6. Overlay Plot of the studied response: Rt for the proportion of acetonitrile and pH of the mobile phase, the mobile phase flow rate was maintained at 1.4 mL min⁻¹**
combinations and interaction of the method parameters that have been demonstrated to respond to the predefined objectives. Any changes performed in the DS can be carried out without risk to the performance of the method. Fig. 8 shows that within the DS the best solution to obtain the best conditions are: %ACN (45.72–46.91); pH (3.8–4.21); A constant flow rate of 1.4 mL min⁻¹.

The desirability function of Derringer. The objective of the present study is to minimize the Rt and maximize RPR and RRA. Therefore, when there are several responses to be optimized with different targets, Derringer’s desirability function (D) is an appropriate technique. The D is defined as the geometric mean, weighted or not, of the individual desirability functions [25, 26]. A value of D different from zero implies that all responses are simultaneously within a desirable range and for a value of D close to 1, the combination of the different criteria is globally optimal, so that the response values are close to the target values. The search for an optimal chromatographic solution was carried out by optimizing various factors to achieve the desired objectives. The desirability diagram Fig. 9 shows that the increase in the %ACN to 46.19% induces a decrease in both resolutions respectively RRA to 13.044 and RPR to 7.20. The pH acts in the same way. This factor should be set at its lowest value. The flow rate, on the other hand, does not have a great effect. For the Rt, the pH has no effect, however the %ACN and the flow rate act negatively. In conclusion, the highest possible composite desirability for our model is 1 and can be obtained under the following conditions: %ACN = 46.19; pH = 3.8; Flow rate = 1.4 mL min⁻¹. In order to facilitate the interpretation of the results, we decided to set the flow rate at 1.4 mL min⁻¹.

Application of the method after optimization. Finally, the study of the development of the HPLC/DAD method for the simultaneous determination of 5 Statins, allowed us to find the following optimal chromatographic conditions: a mobile phase consisting of a sodium acetate buffer solution adjusted to pH = 3.8 (53%) and acetonitrile (46%), a column of...
and RRA. The DS of the method was obtained by having a target Rt of 20 min, RPR and RRA greater than 6 in influencing responses according to the acceptance criteria, ability function was obtained by varying various factors interaction between them. The optimal value of the desirability was discerned by the factor-response relationship and the possible compromise between the %ACN, pH and flow rate. By setting the Rt between 18 and 22 min and RPR, RRA between 6 and 20. The application of the optimized conditions gives very satisfactory results using the column selected at the beginning of our “Waters Spherisorb ODS1” study (250*4mm, 5μm) with a column temperature of 30°C, an isocratic mobile phase containing a buffer (1g sodium acetate pH = 4.13) and acetonitrile (53/46), with a flow rate of 1.4 mL min⁻¹, an injected volume of μL and detection with an iodine strip at 238 nm.

**CONCLUSIONS**

The development and optimization of the procedure is an important and crucial step in the life cycle of an analytical method. The objective of this work is to develop a chromatographic method that allows the simultaneous determination of all five statins. Therefore, during this study a set of chemometric tools are exploited in order to obtain the optimal operating conditions of the optimized method. This work focused on the optimization of an analytical HPLC/DAD assay method. We begin with a screening study of factors influencing the analytical method. This screening was carried out in a univariate manner. From the screening study results, the %ACN, the pH of the buffer solution and the flow rate of the mobile phase were found to be influential factors and were selected for the method optimization study. The retention time (Rt) of the last eluted peak and the resolution between the 3 chromatographic peaks corresponding to RPR and RRA are taken as the response of this optimization study. To find the optimal values of the factors chosen as significant, we choose to work with a 2nd degree compromise between the %ACN, pH and flow rate. By setting the Rt between 18 and 22 min and RPR, RRA between 6 and 20. The application of the optimized conditions gives very satisfactory results using the column selected at the beginning of our “Waters Spherisorb ODS1” study (250*4mm, 5μm) with a column temperature of 30°C, an isocratic mobile phase containing a buffer (1g sodium acetate pH = 4.13) and acetonitrile (53/46), with a flow rate of 1.4 mL min⁻¹, an injected volume of μL and detection with an iodine strip at 238 nm.

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