Rapid development of an LC method for separating high molecular weight degradants from a biopharmaceutical product using an automated Design of Experiments (DOE) approach.

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Introduction

Several LC method development software tools are now available that represent advancements to the traditional one factor at a time (OFAT) approach. However, these tools are limited in terms of (i) number and types of instrument parameters that can be simultaneously studied, (ii) kinds of parameter effects that can be visualized and quantified, (iii) level of system automation that is possible, and (iv) ability to address method robustness. This work describes the use of Fusion AE™, an integrated method development software solution that overcomes all of these limitations, to develop and optimize an accelerated degradation study analytical LC method. The samples mixture comprised a single large biopharmaceutical product peak with several co-eluting degradants and impurities. The legacy LC method used to separate these components comprised a two-step gradient method that did not meet all method performance requirements in terms of peak resolution, total assay time, and overall robustness. In the legacy method the active pharmaceutical ingredient (API) peak remained unresolved from peaks on either side, thereby complicating quantitation and peak purity determinations (Fig 1).

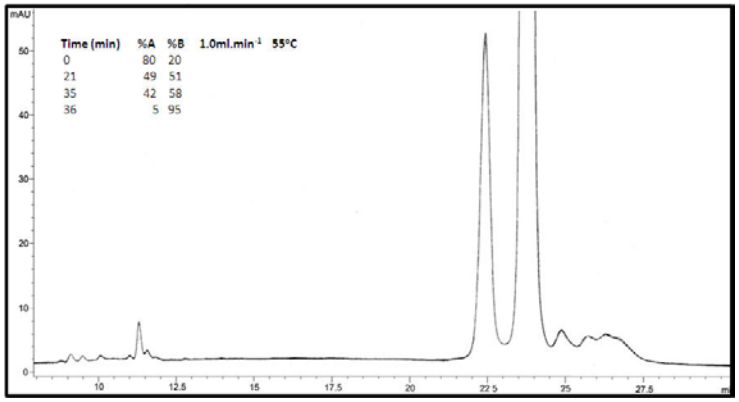


Figure 1. Incomplete separation of API from degradant and impurity peaks using the legacy two step LC gradient method

Materials and Methods

Method Development LC System:

Agilent 1100 HPLC (Agilent Corp., Palo Alto, CA.). G1312 Binary Pump, G1313 Autosampler, G1314 Variable Wavelength Detector & G1316 Column Compartment

LC system control and data processing:

Galaxie™ chromatography data system, (Varian Inc., Palo Alto, CA.).

Experimental design, data analysis, modeling, method optimization:

Fusion AE Method Development Software Platform (S-Matrix Corp., Eureka, CA.)

Column:

150 x 4.6 mm PLRP-S 1000A 8u (Polymer Labs)

Mobile Phase:

Water/TFA (0.1% v/v), Acetonitrile/TFA (0.1% v/v)

Sample Conditions:

40 weeks storage at pH 6.5, 54^{o}C

Experimental

Initial screening experiment – The study variables and ranges for this experiment are shown in Table 1. These were studied according to a 27-run statistical experimental design generated by Fusion AE. The design included repeat injections at three different experimental conditions for experimental error estimation and 5 "Lack-of-Fit" degrees-of-freedom runs to support correct equation building. Fusion AE's export operation automatically reconstructed this experimental design in the CDS as ready-to-run LC methods and sequence. The experiment was run overnight on the HPLC in walk-away mode.

Table 1. Screening experiment 1 study variable and ranges

Pump Flow Rate (ml/min)	0.5 -1.5
Gradient Time (min)	10 - 50
Gradient Slope (Final % B)	40 - 95
Column Oven Temperature (^{o}C)	40 - 65

Fusion AE imported the chromatographic results from the CDS via a file-less data exchange, and then automatically analyzed the results to create a prediction equation for each result. The Automated Optimizers then carried out optimum solution searches numerical and graphical optimizers using the user-defined goals presented below. The Numerical Optimizer identified the optimum method presented in Table 2, with corresponding predicted results for the three critical peaks presented in Table 3.

- USP Resolution of API (Peak 2) from potentially co-eluting impurities (Peaks 1 and 3): ≥1.5
- Total Assay Time (set by minimizing Retention Times of last-eluting peaks): ≤ 30 minutes.

Table 2. Automated Optimizer Predicted Optimum Method

Study Variable Name	Optimizer Answer Level Setting
Pump Flow Rate	1.41
Gradient Time	10.0
Gradient Slope	66.8
Oven Temperature	40.0

Table 3. Automated Optimizer Predicted Results for Predicted Optimum Method

Response Variable Name	Target	Optimizer Answer Predicted Response	-2 Sigma Confidence Limit	+2 Sigma Confidence Limit	Relative Rank
Peak_1 - Peak Retention Time	Minimize	6.72	5.87	7.57	1.0
Peak_2 - Peak Retention Time	Minimize	7.96	6.16	9.76	1.0
Peak_2 - Resolution	Maximize	1.76	1.26	2.26	1.0
Peak_3 - Peak Retention Time	Minimize	7.12	5.28	8.97	1.0
Peak_3 - Resolution	Maximize	2.59	2.27	2.92	1.0

Figure 2 presents an Overlay Graph generated by Fusion AE using the equations derived from analysis of the initial screening experiment results. In this graph each peak resolution and retention time goal is assigned a color by the software, and the graph region shaded by that color shows all Gradient Time (x-axis) and Final % B (y-axis) combinations which do not meet the corresponding goal. The un-shaded region therefore corresponds to Gradient Time and Final % B combinations which exceed all defined goals. Note that this graph was generated by setting the *non-graphed* variables Pump Flow Rate and Column Oven Temperature to their optimum levels as defined by the optimum solution search result (see Table 2).

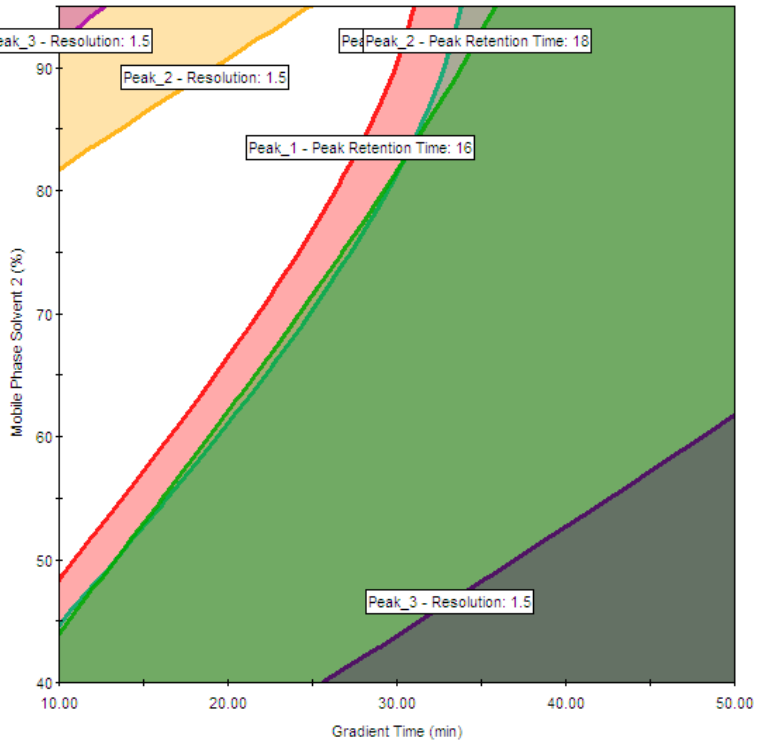


Figure 2. Overlay graphic showing extensive region where separation goals were met in initial screening experiment.

Secondary screening experiment – Analysis of the initial screening experiment data defined the optimum Final %B and acceptably separated all later-eluting compounds. As a result, a second screening experiment was designed and carried out in which the Final %B was set to a constant at the optimum level and which varied the Initial %B to optimize the separation of the earlier-eluting peaks. Study variable and ranges for this experiment are shown in Table 4. These were varied according to a 27-run screening experimental design generated by Fusion AE. As before, the experiment was run overnight on the HPLC in walk-away mode. Chromatographic results were then imported from the CDS into Fusion AE and automatically analyzed.

Table 4. Screening experiment 2 study variable and ranges

Pump Flow Rate (ml/min)	0.5 -1.5
Gradient Slope (Initial % B)	20 - 45
Gradient Time (min)	10 - 50
Column Oven Temperature (^{o}C)	40 - 65

Following data analysis, robustness metrics for each method performance requirement were automatically computed and analyzed for each experimental method by Fusion AE's patented Robustness Simulator™.

Optimization solution searches were then conducted using the software's numerical and graphical optimizers using the following user-defined goals.

- USP Resolution of API (Peak 2) from co-eluting impurities (Peaks 1 and 3): ≥ 2.0
- USP Tailing: Target, 0.90 ≤ Tailing ≤ 1.50
- Peaks 2 and 3 Resolution Robustness: Maximize, ≥ 1.33

Note that a lower limit value of 1.33 has been defined for the Resolution Robustness goal. This value imposes the requirement that the ±3σ variation in a critical pair Resolution result occurring on method transfer and normal use over time will encompass at most 75% of the acceptable variation range. In other words, the acceptability limits bracketing the expected Resolution result will be located at the ±4σ variation limits of the result (4/3 = 1.33). In practical terms this means that the method should never yield a critical pair Resolution result outside the acceptable performance limits on transfer.

The Numerical Optimizer searches identified the optimum method presented in Table 5, with corresponding predicted results for the three critical peaks presented in Table 6. As the table shows, the final method exceeded all performance goals for critical pair Resolution, Retention Time (assay speed), and Resolution Robustness.

Table 5. Automated Optimizer Predicted Optimum Method

Study Variable Name	Optimizer Answer Level Setting
Pump Flow Rate	1.1
Gradient Time	44.0
Initial % B	37.5
Oven Temperature	55.0

Table 6. Automated Optimizer Predicted Results for Predicted Optimum Method

Response Variable Name	Target	Optimizer Answer Predicted Response	-2 Sigma Confidence Limit	+2 Sigma Confidence Limit	Relative Rank
Peak_1 - Tailing Factor	1.2	1.04514	0.94050	1.14978	1.0
Peak_2 - Resolution	Maximize	2.12333	1.98758	2.25908	1.0
Peak_2 - Tailing Factor	1.2	1.47298	1.39656	1.54939	1.0
Peak_3 - Resolution	Maximize	2.03589	1.56255	2.50923	1.0
Peak_3 - Tailing Factor	1.2	0.90006	0.76590	1.03421	1.0
Peak_4 - Resolution	Maximize	0.99998	0.71227	1.28770	1.0
Peak_4 - Tailing Factor	1.2	1.19084	0.81224	1.56945	1.0
Peak_2 - Resolution - Robustness	Maximize	2.170012	2.090255	2.252813	1.0
Peak_3 - Resolution – Robustness	Maximize	2.161595	2.158550	2.164643	1.0

Figure 3 presents an Overlay Graph generated by Fusion AE using the equations derived from analysis of the experiment results. In this graph the *non-graphed* variables Initial % B and Gradient Time were set to their optimum levels as defined by the optimum solution search result (see Table 5). Note that the un-shaded region in the figure encompasses the Automated Optimizer answer.

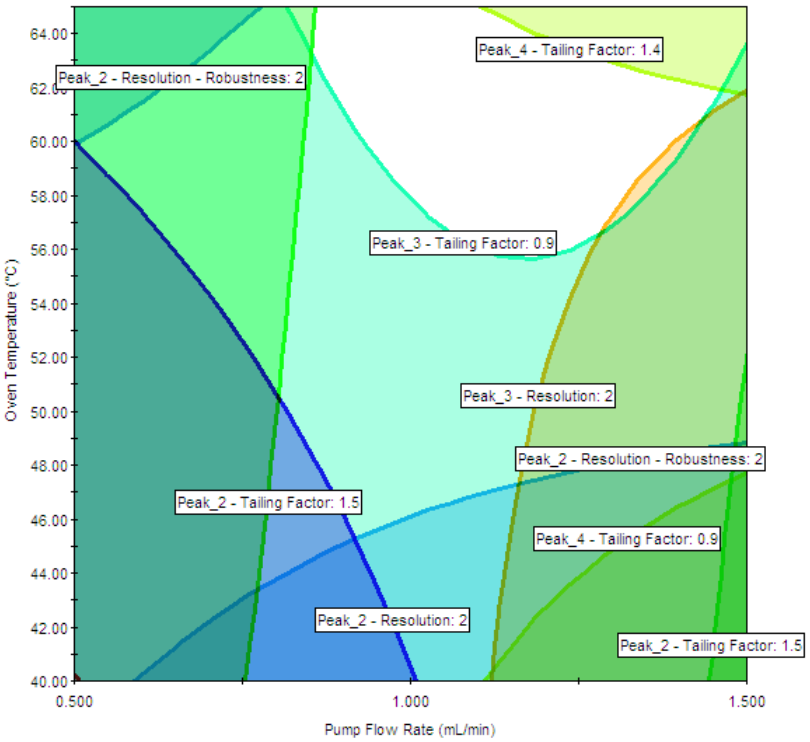
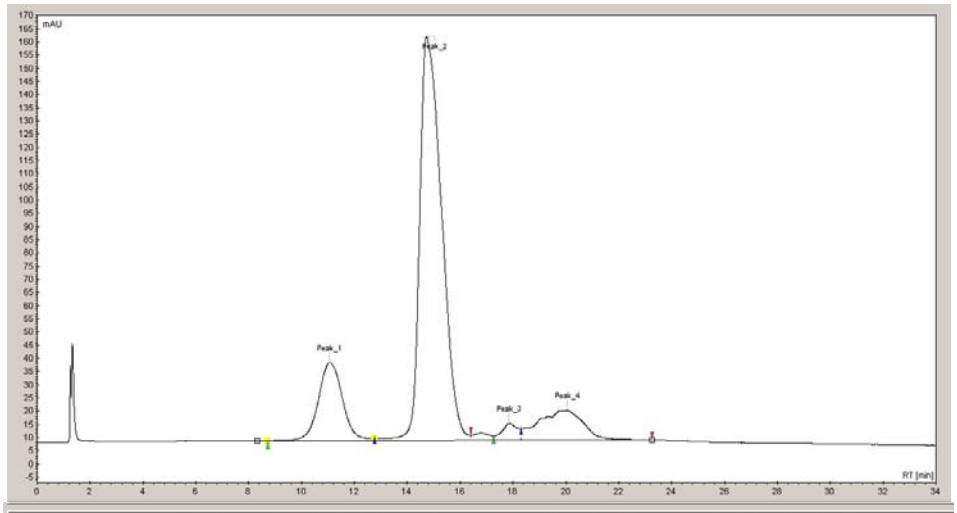


Figure 3. Overlay graphic showing extensive region where separation goals were met in secondary screening experiment.

Figure 4 presents the chromatogram obtained by constructing a method using the Automated Optimizer search result settings of the experiment variables defined in Table 5. Note that all peaks are baseline resolved in the chromatogram, and that the critical impurities are well separated from the API. Note also that all peaks are eluted before 24 minutes in this chromatogram, defining a total required assay time of below 30 minutes. The total time required for this method development project was one week.



#	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]	Res. USP	As. USP
1	Peak_1	11.06	15.16	29.6	31.1	15.158	0.00	1.07
2	Peak_2	14.73	71.81	153.2	147.1	71.805	2.35	1.56
3	Peak_3	17.85	2.18	6.4	4.5	2.176	2.54	0.88
4	Peak_4	20.05	10.86	11.3	22.3	10.860	1.16	0.98
Total			100.00	200.4	204.9	100.000		

Figure 4. Prediction chromatogram from method run at point prediction setting and associated peak results table.

Results

1. Quality-by-Design (QbD) principles governed the experimental approach, and Design of Experiments (DOE) methodology was used in constructing the experimental designs.
2. Critical chromatographic performance metrics (responses) were statistically analyzed, and all equations (models) fit the data – all model coefficients ware statistically significant, and model prediction error ≈ experimental error.
3. Resolution Robustness metrics were computed for each optimization experimental run using a modified Monte Carlo simulation approach that employs (a) the Resolution models obtained from analysis of the experimental results, (b) specified variations in the LC instrument parameters studied, and (c) user-defined acceptable variation limits for each critical response.
4. The following specified method performance goals were met:
    a. USP Resolution – 2.0 ±0.25
    (higher resolutions may cause problems with adjacent later-eluting peaks)
    b. Resolution Robustness – ≥1.33 or all critical peak pairs.
5. Gradient Program – a one-step gradient with a total assay time of ≤ 30 minutes.

Conclusions

Fusion AE was able to successfully develop an HPLC method optimized for flow rate, gradient time, gradient slope and column temperature which met all critical method performance requirements. Response surface plots graphically illustrate the major effects of flow rate and column temperature on resolution of the critical peaks. Further, due to synergistic interaction effects of these parameters, optimum performance is shown to be achievable at medium flow – high temperature combinations. The Overlay Graphics plot additionally illustrates the high robustness of the predicted optimum method with respect to variations in the study parameters. The rigorous experimental approach provided a rich data set which, when combined with the chromatogram obtained from running the predicted optimum method, provide both experimental and statistical defensibility for the defined final method.

Literature Cited

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