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Quality by Design: Multidimensional exploration of the design space in high performance liquid chromatography method development for better robustness before validation

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ABSTRACT

Robust HPLC separations lead to fewer analysis failures and better method transfer as well as providing an assurance of quality. This work presents the systematic development of an optimal, robust, fast UHPLC method for the simultaneous assay of two APIs of an eye drop sample and their impurities, in accordance with Quality by Design principles. Chromatography software is employed to effectively generate design spaces (Method Operable Design Regions), which are subsequently employed to determine the final method conditions and to evaluate robustness prior to validation.

1. Introduction

Chromatography modeling software has been widely and successfully used for many years in the pharmaceutical industry for developing robust high performance liquid chromatography (HPLC) methods [1–7]. The workflow typically adhered to begins HPLC method development with predefined goals, focuses on chromatographic understanding [8], is based on retention models which provide a solid scientific foundation [9–11] and can be identified, therefore, as a Quality by Design (QbD) approach [12,13]. As well as modeling software, other computer programs providing calculations of lipophobicity, log *P*, log *D* and pKa values have also been found to be useful tools in the method development process [14–17]. In this study, both aids to robust method development are employed within a QbD framework.

Recently, a number of excellent articles have been published describing general strategies for the application of QbD principles to analytical measurements [18,19] and to the development of HPLC methods [20,21]. Four key steps, as shown in Fig. 1, are commonly described: first, objectives of the method or the target method performance criteria are clearly defined (method intent); second a design and selection of the method takes place (method design, also referred to as method scouting); thirdly the selected method is thoroughly assessed (method evaluation) and finally a control strategy is implemented (method control). The present work addresses each of these key steps with a special focus on HPLC method robustness.

A fundamental criteria of quality in an HPLC separation, is robustness. Current guidelines define the robustness of an analytical procedure as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. . ." providing ". . .an indication of its reliability during normal usage" [22]. In the past, robustness testing was typically carried out during the final stages of a method development process during the validation stage [23] which often led to undesired surprises being found late on and the method having to be redeveloped and reoptimized. To avoid these costly repetitions, there is an increasing tendency to include thorough, multifactorial robustness evaluations at an early stage of development [24], to build in quality from the outset. By defining method operating conditions not as discrete points but rather as working spaces with known tolerances, the flexibility of a method is increased and the likelihood of method failure is reduced, as the method can withstand small changes, by design.

A modern, QbD based treatment of the robustness of an HPLC method requires the assessment of all parameters (factors) which most strongly influence selectivity (results) alone and in combination. In the majority of cases, these so-called critical parameters are gradient time (t_G), temperature (T), pH of eluent A (pH), ternary eluent composition (tC), and stationary phase. Notwithstanding, other parameters such as flow rate, start %B, end %B, dwell volume, etc., may also be important in gradient elution. The experimental verification of many factors simultaneously is impractical and associated

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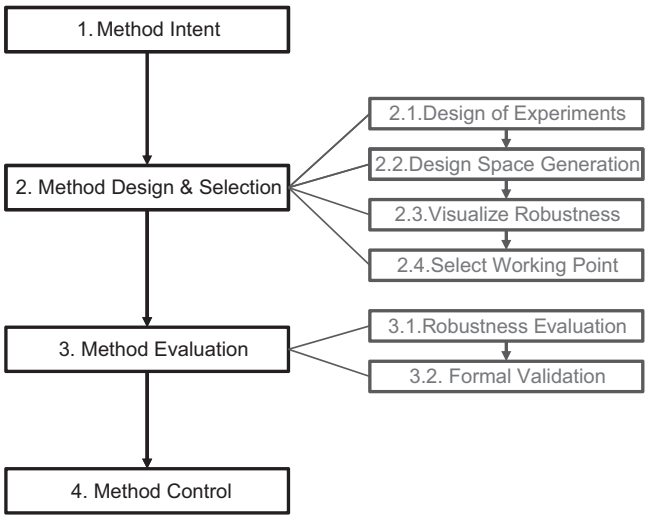


Fig. 1. Workflow followed in present study.

with extreme technical difficulties and expense. Some authors, to overcome the challenge and reduce the experimental workload have employed statistical studies, such as Plackett–Burman or fractional factorial designs [25–27] and risk-based approaches [28]. Other procedures include running automated robustness experiments [29,30]. The present article, however, employs modeling – rather than statistical analysis or automated execution of hundreds of experiments – to evaluate robustness.

In HPLC analysis, for a method to be accurate, precise and robust the sample must first be well separated. Therefore a prerequisite for robustness can be established, in that the critical resolution ($R_{s,crit}$) – resolution between the least well separated peak pair – for all peaks of interest must be above a threshold value (for example, for baseline separation $R_{s,crit} \geq 1.5$). In this way, all conditions for which the $R_{s,crit}$ remains above a given value (e.g. 1.5 and 2.0) are robust. A number of different studies have used resolution maps, which plot critical resolution against critical method parameters, to study and define robustness [31–34]. This study continues along the lines of these works, and employs novel features in the modeling software DryLab® to visualize and evaluate the effect on separation selectivity of varying a relatively large number of parameters simultaneously. This approach to robustness evaluation is thought to exhibit a number of advantages: the robustness of a separation at a given working point can be more thoroughly assured, the experimental workload is greatly reduced, the excessive need for statistical analysis is eliminated and the size of tolerance windows for each parameter can be assessed and determined scientifically at the stroke of a key.

2. Experimental

2.1. Eluents and reagents

Acetonitrile (AN) (Merck, Darmstadt, Germany) and methanol (MeOH) (LABSCAN, POCH S.A., Gliwice, Poland) were gradient grade and water was highly purified MilliQ (Merck Millipore).

Eluent A was 50 mM KH_2PO_4 (analytical grade, Merck, Darmstadt, Germany) in water:AN (95:5, v/v). The pH was adjusted to pH 2.1, pH 2.7, pH 3.3, pH 6.8, pH 7.4 and pH 8.0 prior to adding AN. In the acidic pH range the pH was adjusted with 85% H_3PO_4 and in the alkaline pH range the pH was adjusted with 0.2 M NaOH. Eluent B were solutions of 50 mM KH_2PO_4 in water:AN (20:80, v/v), 50 mM KH_2PO_4 in water:AN:MeOH (20:40:40, v/v/v) and 50 mM KH_2PO_4 in water:MeOH (20:80, v/v). For the sake of simplicity, these eluent B compositions will be abbreviated to AN, AN:MeOH (1:1, v/v) and MeOH, respectively, from this point onwards.

2.2. Equipment

HPLC separations were performed on a Waters Acquity UPLC® System (Waters, Milford, MA, USA) equipped with (binary solvent pump with a vacuum degasser, PDA detector, cooled autosampler, temperature controlled column compartment). The dwell volume of the instrument was measured to be 0.12 mL and the extra column volume was estimated at 2 μL. Data acquisition was performed using the Waters Empower 2 chromatography data software. All separations were carried out using a Waters HSS T3 C18 column (100 mm × 2.1 mm, 1.8 μm) supplied by Waters (Budapest, Hungary).

2.3. Sample

The sample used throughout the study was an eye drop solution containing 2 APIs (Active Pharmaceutical Ingredient) – A and B – and 9 known impurities (4 API-A impurities and 5 API-B impurities) each spiked at 0.1% level of their respective API. The 2 APIs were dissolved in water at concentrations of 0.04 mg/mL API-A and 0.137 mg/mL API-B. The 9 impurities were dissolved in 1 mL AN and 9 mL water. Further dilutions were made with water.

2.4. Software

2.4.1. Chromatography modeling and prediction software

Modeling was carried out using DryLab®2010 v.4.0 (Molnár-Institute, Berlin, Germany) and the quantitative robustness evaluation of generated models was performed in the latest DryLab® Robustness Module v.1.0.

2.4.2. Chemical expert software

Marvin® v. 5.5.1 (ChemAxon Kft, Budapest, Hungary) was employed for the generation of log *D* diagrams.

2.4.3. Column comparison software

Column equivalence was investigated in the database ColumnMatch® (Molnár-Institute, Berlin, Germany).

2.5. Experiments for modeling

Three different 3D resolution models were constructed: [Cube A] t_G–*T*–pH cube at acidic pH, using AN as eluent B, [Cube B] t_G–*T*–pH cube at neutral pH, using AN as eluent B and [Cube C] t_G–*T*–tC cube, using eluent A with pH 2.7. The conditions of the modeled parameters for the input runs, selected largely in compliance with recommendations from Snyder et al. [1], can be found in Table 1 according to the design of experiments shown in Fig. 3. The flow rate – 0.3 mL/min – and gradient range – 0 → 100%B – remained constant throughout the systematic work.

3. Results and discussion

The workflow adhered to in this study – leading to the final method conditions (Section 3.4) – is schematically represented in Fig. 1 and detailed below.

3.1. Method intent

The aim of this study was to develop a fully validated UHPLC method in accordance with QbD principles for the assay of two APIs (API-A and API-B) and impurities for an eye drop sample, providing a fast and robust stability indicating analysis.

Table 1
Summary of experimental conditions of measured parameters necessary for the generation of 3D resolution models.

Cube	t_G (min)		T (°C)		pH			tC		
	t_{G1}	t_{G2}	T_1	T_2	pH_1	pH_2	pH_3	tC_1	tC_2	tC_3
[A] t_G–T–pH (acidic)	15	30	25	50	2.1	2.7	3.3	AN		
[B] t_G–T–pH (neutral)	15	30	25	50	6.8	7.4	8.0	AN		
[C] t_G–T–tC	15	30	25	50		2.7		AN	AN:MeOH	MeOH

In order to generate accurate and precise data all impurities must be separated from each other and from the main peaks, therefore method target performance criteria was baseline or better separation for all peaks within a robust working region. Additionally, all acceptance criteria pertinent to a formal validation [22] should be met (see Appendix A).

3.2. Method design and selection

3.2.1. Design of experiments

Based on prior knowledge and a risk-based assessment, the critically influential separation parameters gradient time, temperature, pH of eluent A, stationary phase and ternary eluent composition were selected for primary systematic and scientific evaluation.

Column selection and best pH working range were deduced from log D diagrams (Fig. 2) depicted by the Marvin® chemical expert program on the basis of the molecular structure and physicochemical properties of the sample constituents.

As seen in Fig. 2 the values of log D are relatively robust (flat slope) with regards to the pH in two regions of the diagram: one at low pH around pH 2–4 and another at high pH around pH 11–13. This would suggest that retention times of sample analytes should remain largely constant with regards to the pH in these ranges. In the pH range 6–8 log D curves for the individual compounds change quite drastically with the pH, indicating that in this region peak retention values would be more sensitive to small changes in the pH. Though, a priori, either one of the two predicted robust pH ranges may have been selected for method development, the acidic

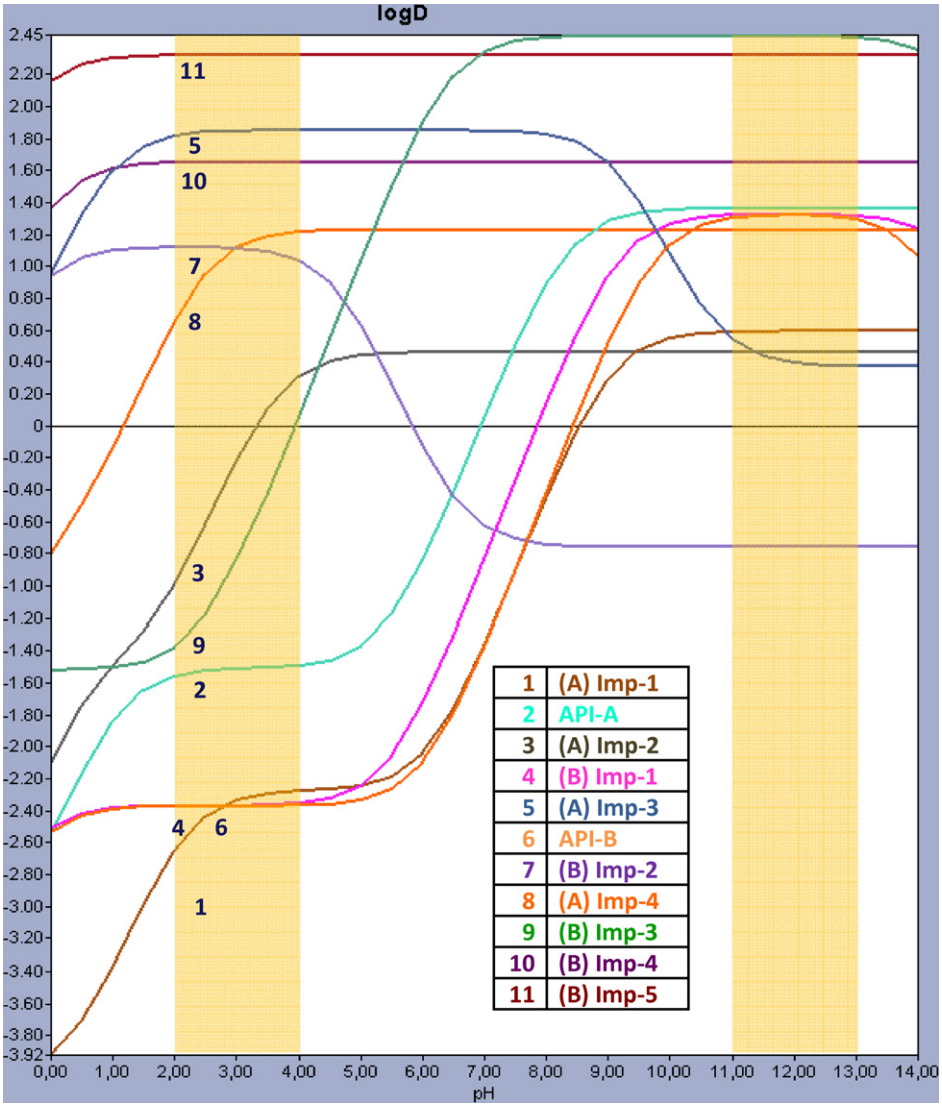


Fig. 2. Marvin® log D diagrams for each constituent of the eye drop sample.

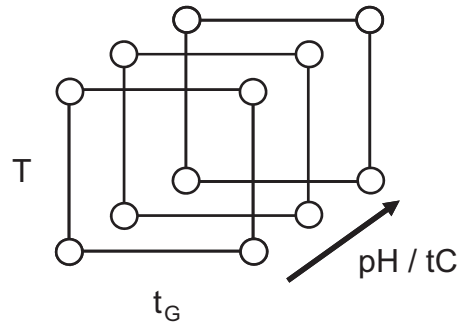


Fig. 3. Design of experiments for the construction of 3D resolution spaces.

pH region (2–4) was chosen for this study, rather than the alkaline range (11–13). It can also be seen that in the acidic pH range, the log *D* values for most components is below 0 indicating that compounds contained in the eye drop sample are very polar. For this reason a Waters HSS T3 column, suitable for the separation of polar compounds and compatible with high water content, was selected. The significant differences in log *D* values furthermore indicated that gradient elution would be appropriate for this separation.

From the primary analysis of log *D* data, a formal design of experiments (DoE) was carried out for the modeling of the retention behavior of the separation, employing the chromatography software DryLab®2010. First the influence on relative retention of gradient time, temperature and pH of eluent A – in the acidic pH range – were investigated in a simultaneous fashion by means of 3D resolution cubes (Cube A). To verify the decision to work at acidic pH, another t_G–*T*–pH cube was generated in the neutral pH range (Cube B). Once an optimal acidic pH was determined, a further 3D resolution cube modeling gradient time, temperature and ternary eluent composition was constructed (Cube C) at optimal pH. Parameter settings and ranges were selected in accordance with the software's guidelines and the generic DoE is shown in Fig. 3.

3.2.2. Design space generation/Method Operable Design Region (MODR) determination

Following the execution of the input experimental runs, data were imported into DryLab®2010, peak tracking was performed and models were constructed. As two different wavelengths were used for detection, retention times and peak areas (taken as peak area average values of both wavelengths) were keyed directly into the software, upon which peak tracking – the matching of bands for the same compound between runs where conditions have been changed – was carried out.

The resulting resolution space for Cube A modeling gradient time, temperature, low pH and critical resolution (color) is shown in Fig. 4. Resolution models map the critical resolution for each combination of the study parameters (i.e. t_G, *T*, pH, ternary). The value of the critical resolution ($R_{s,crit}$) is represented in color so that warm colors show large $R_{s,crit}$ values and cold colors show low values corresponding to inefficient separations. Specifically, in red regions the resolution is baseline or above ($R_{s,crit} \geq 1.5$) and dark blue lines signalize peak overlaps ($R_{s,crit} = 0$).

3.2.3. Visualization of robustness

When all working points (i.e. combinations of measured parameters) with a critical resolution below the threshold of 1.5 ($R_{s,crit} < 1.5$) are removed from the resolution spaces, robustness regions can be identified and the robustness of the separation can be visualized as irregular geometric bodies. The robustness space for Cube A and Cube B are shown in Fig. 5.

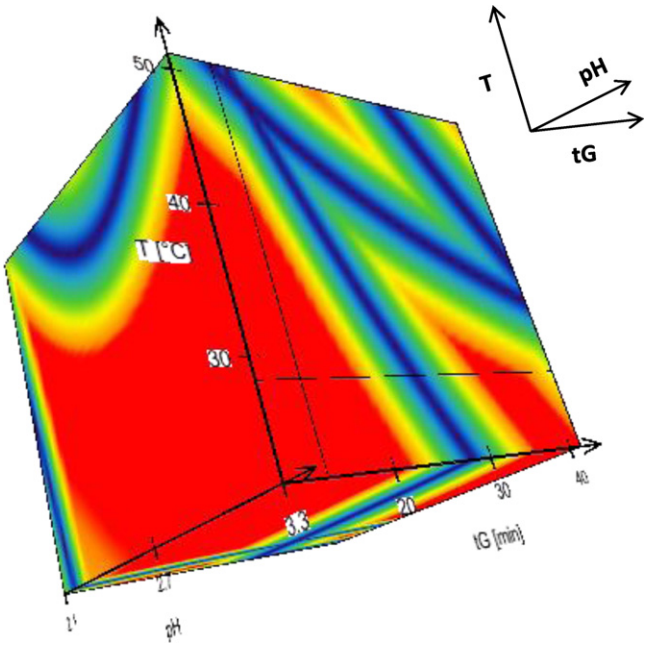


Fig. 4. 3D resolution space for Cube A mapping the simultaneous influence of gradient time (t_G), temperature (*T*) and pH on critical resolution (color). Regions yielding above base-line resolution ($R_{s,crit} > 1.5$) are colored red.

3.2.4. Selection of working point/method selection

The working point was selected on the basis of two criteria: it should be contained within the largest robustness space (center of the red region) and have the shortest run time. This point was found to be within Cube A at t_G: 7 min, *T*: 25 °C and pH 2.7, a position located in the center of the "best" robustness space to guarantee the largest possible tolerances. The predicted and experimental verification of this chromatogram are shown in Fig. 6.

The accuracy of the model generated, as also reported in the literature [5,9–11,32,35] was found to be excellent.

3.3. Method evaluation

Next, risk assessment was performed and the robustness and ruggedness of the selected working point was evaluated. Robustness was studied with the aid of the generate DryLab® models and ruggedness was assessed as part of the formal validation process.

3.3.1. Multifactorial robustness study

3.3.1.1. Robustness of measured parameters – t_G, T, pH. Within Cube A the three measured parameters' tolerances were determined to be t_G: 7 ± 1 min, *T*: 25 ± 2 °C and pH: 2.7 ± 0.1 by visual inspection of the 3D resolution model. To confirm these tolerances, 12 new experiments were carried out (Table 2), all of which gave a good separation – critical resolution of 1.5 or better – of all peaks of interest. As a further measure of model verification, the 12 chromatograms were used to construct a new, smaller cube, which, as predicted, proved to yield critical resolution above 1.5 in the whole range.

3.3.1.2. Robustness of measured and calculated parameters – t_G, T, pH, flow rate, start %B, end %B. As it is possible to mathematically calculate the influence of flow rate, start %B, and end %B on retention times, tolerance windows of these factors were ascertained within the DryLab® software without the need for further experimentation, with the aid of the novel Robustness Module (Fig. 7). This module uses simulated data from created models to carry

Table 2
Experimental conditions and verification of robustness tolerances surrounding selected working point. Only the critical region of the chromatograms is shown.

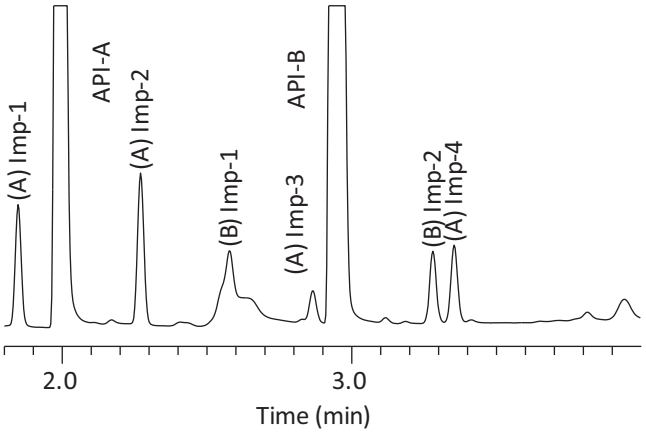
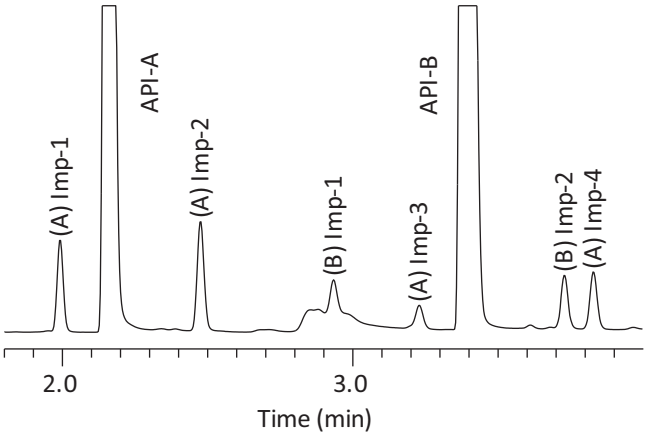
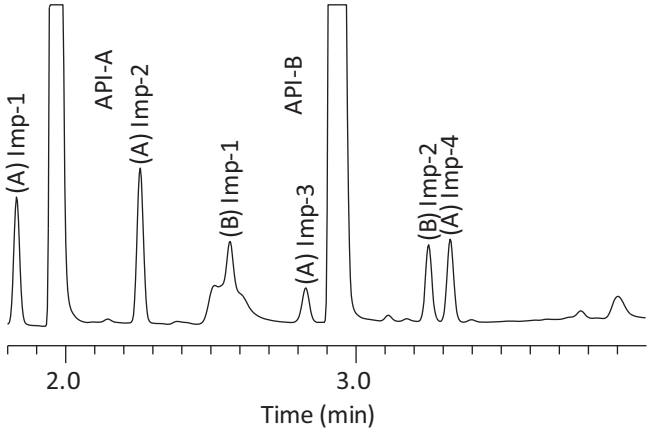
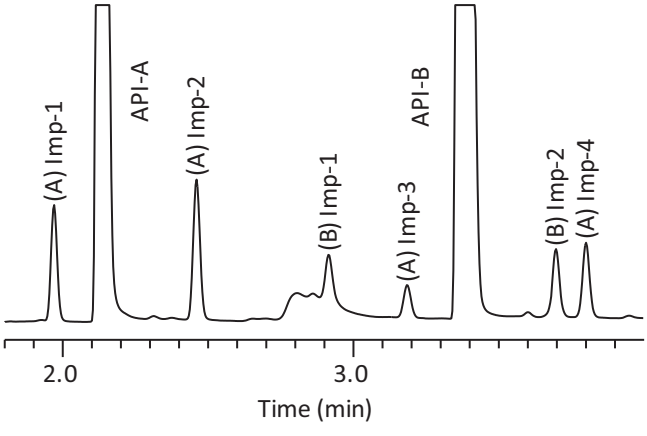
Exp. no.	t_G (min)	T (°C)	pH	Experimental chromatogram
Exp. 1	6	23	2.6	
Exp. 2	8	23	2.6	
Exp. 3	6	27	2.6	
Exp. 4	8	27	2.6	

Table 2 (*Continued*)

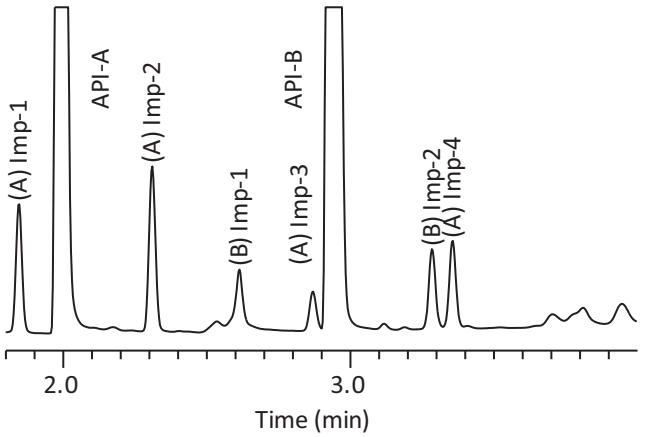
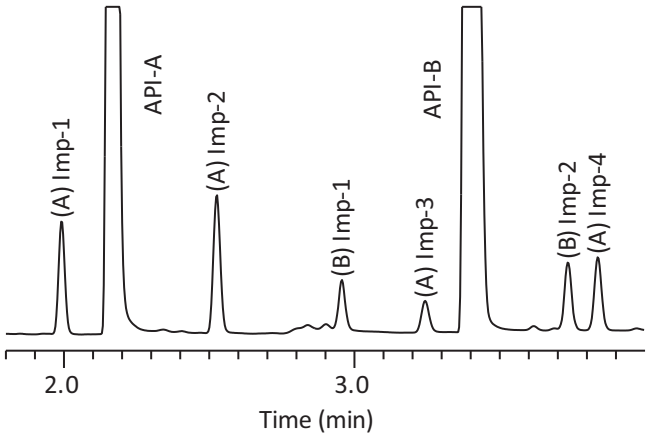
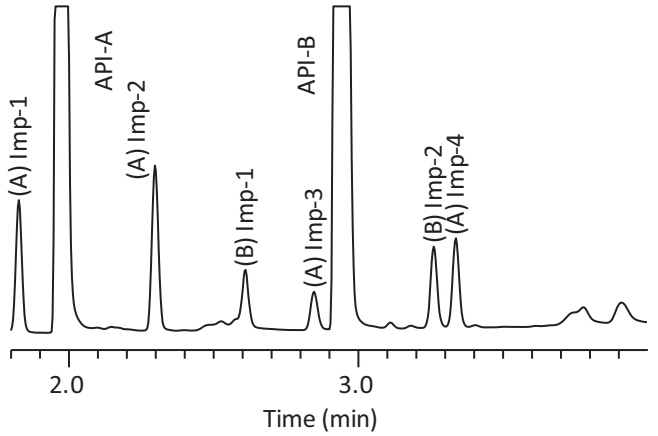
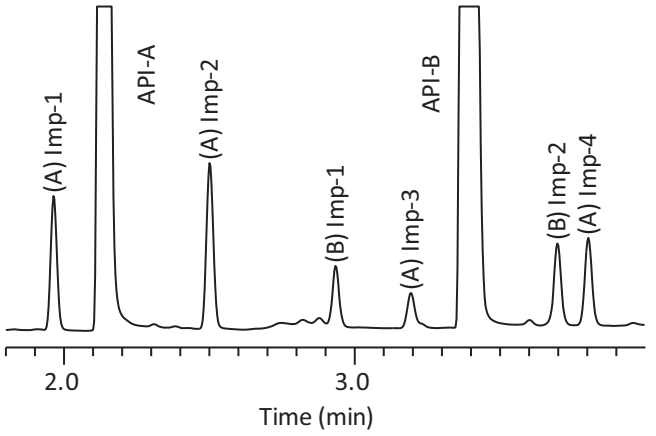
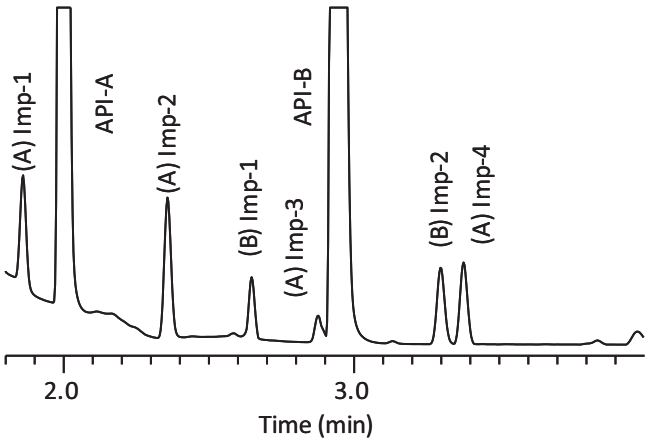
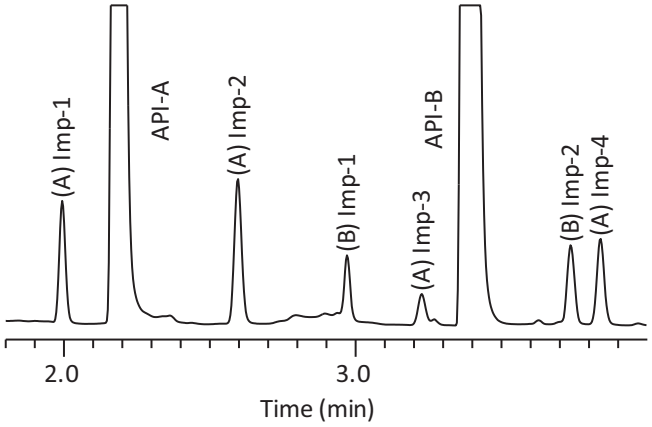
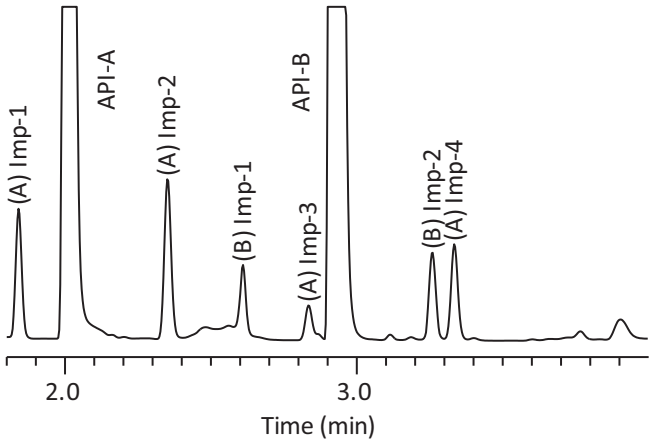
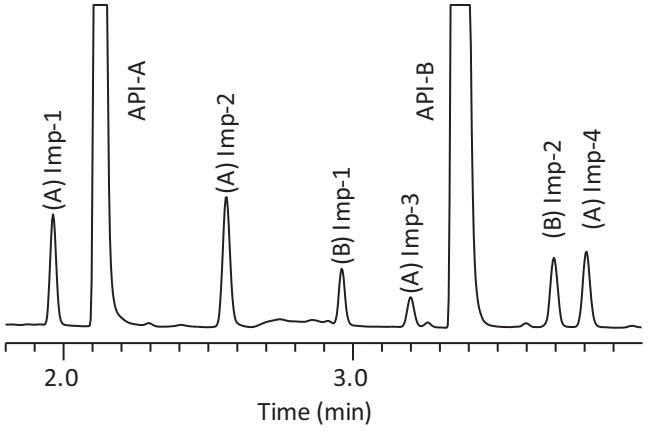
Exp. no.	t_G (min)	T (°C)	pH	Experimental chromatogram
Exp. 5	6	23	2.7	
Exp. 6	8	23	2.7	
Exp. 7	6	27	2.7	
Exp. 8	8	27	2.7	

Table 2 (*Continued*)

Exp. no.	t_G (min)	T (°C)	pH	Experimental chromatogram
Exp. 9	6	23	2.8	
Exp. 10	8	23	2.8	
Exp. 11	6	27	2.8	
Exp. 12	8	27	2.8	

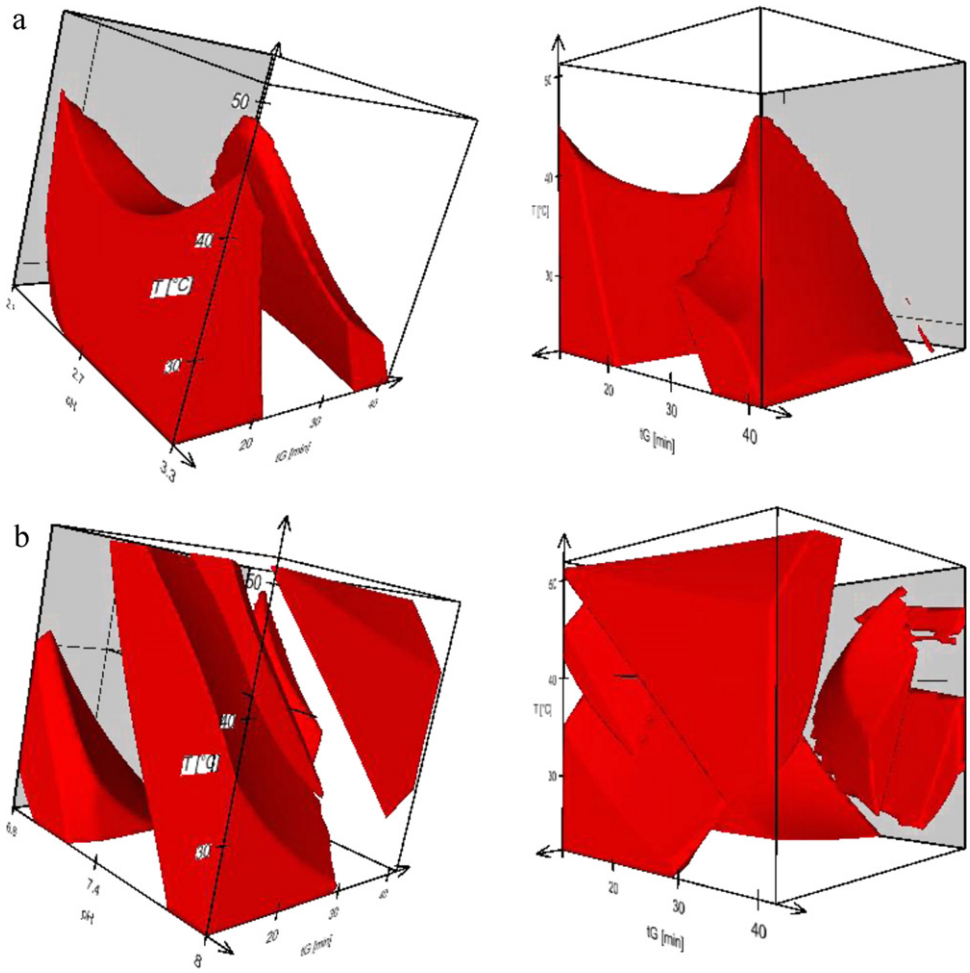


Fig. 5. Robustness space for: (A) Cube A and (B) Cube B from two different angles. Red regions represent robust above baseline separations.

out full-factorial robustness evaluations, registering responses including critical resolution ($R_{s,crit}$) critical peak pairs and run time.

This extended multifactorial robustness testing consisted of keying in the nominal values for t_G, T, pH, flow rate, start %B and end %B, i.e. the conditions of the selected working point; typing in different tolerance windows for each parameter, upon which a full-factorial design of experiments is automatically generated; and finally initiating the calculation of the responses (calculation time < 1 min). For this study, a number of different tolerances were evaluated for the flow rate, start %B and end %B, in combination with the previously defined "± values" of t_G, T and pH. Results of this 6D analysis are shown in Table 3.

In test (a) the critical resolution and critical peak pair are shown for the selected discrete working point, in the absence of tolerances. Test (b) introduces relatively small tolerance windows, which increase over tests (c) and (d). Upon amplifying the magnitude of the tolerance windows: the average value of critical resolution decreases gradually, the range between highest and lowest ($R_{s,crit}$) values increases and the number of critical peak pairs also augments. This is due to an expansion of the multi-dimensional parameter space around the working point being investigated. In test (d) the $(R_{s,crit})_{min}$ falls below the method criteria of above baseline separation, therefore the evaluated tolerances are too large for this analysis. The final tolerances selected for this study were those indicated for test (c), as they are larger than the instrument

Table 3
Summary of robustness calculations for different tolerance windows around the selected working point.

	Parameter ± tolerance						Results/responses			
	t_G (min)	T (°C)	pH	Flow rate (mL/min)	Start %B (%)	End %B (%)	$(R_{s,crit})_{avg}$ [a]	$(R_{s,crit})_{max}$ [b]	$(R_{s,crit})_{min}$ [c]	Crit. PP[d]
[a]	7	25	2.7	0.3	0	100	2.14	2.14	2.14	(7,8)
[b]	7 ± 1	25 ± 2	2.7 ± 0.1	0.3 ± 0.01	0 + 0.1	100 − 0.1	2.14	2.35	1.93	(7,8) (5,6)
[c]	7 ± 1	25 ± 2	2.7 ± 0.1	0.3 ± 0.05	0 + 0.5	100 − 0.5	2.12	2.60	1.67	(7,8) (5,6)
[d]	7 ± 1	25 ± 2	2.7 ± 0.1	0.3 ± 0.1	0 + 1	100 − 1	2.10	2.88	1.28	(7,8) (5,6)

[a] $(R_{s,crit})_{avg}$ = sum $R_{s,crit}$ values $(\Sigma R_{s,crit})_i$/no total $R_{s,crit}$ values (N_i). $N_i = \prod$no levels$^{\text{no factors}} = 3^4 \times 2^2 = 324$.
[b] $(R_{s,crit})_{max}$ = highest value of critical resolution found within the 324 experiments.
[c] $(R_{s,crit})_{min}$ = lowest value of critical resolution found within the 324 experiments.
[d] crit. PP = critical peak pairs.

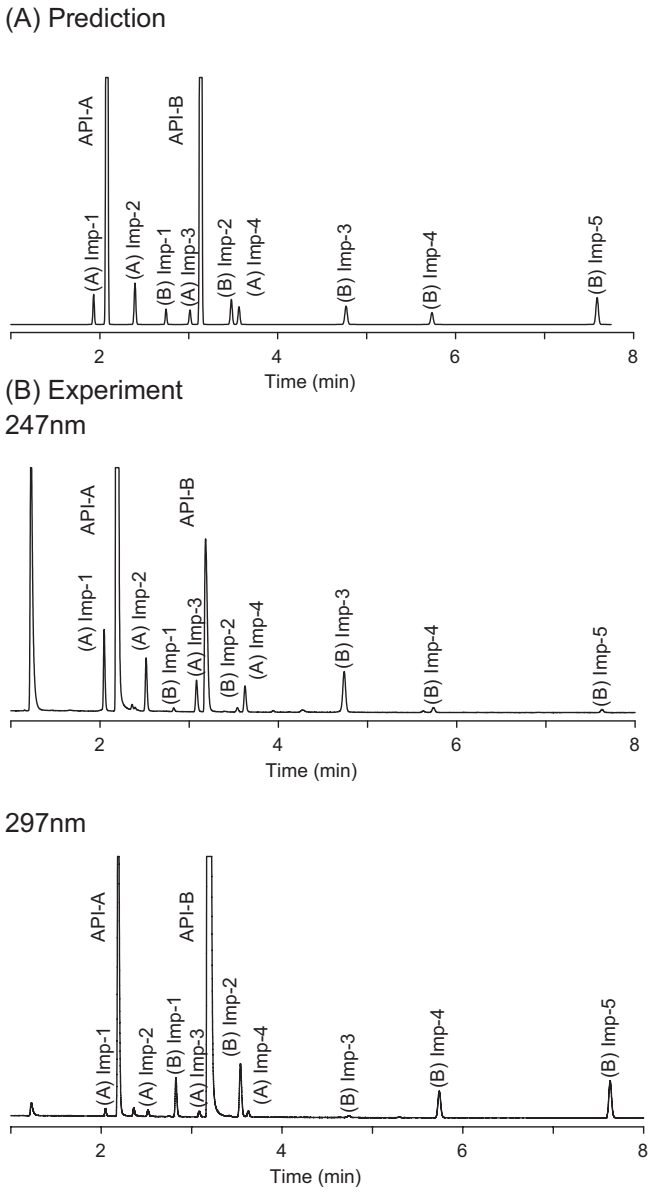


Fig. 6. Comparison between predicted (A) and experimental (B) chromatogram for the working point t_G: 7 min, T: 25 °C, pH: 2.7.

precision [36] and allow for the largest experimental tolerance without compromising the separation.

3.3.1.3. Robustness of column chemistry. The stationary phase is another critically influencing parameter in HPLC and was also included in the robustness evaluation. In the first place, models were constructed with a theoretically lower plate number than the experimentally observed, in order to take into account the inevitable loss of column performance over the lifetime of the column. Secondly, the ColumnMatch® [37–40] software (Fig. 8) was employed to investigate column equivalence. Three columns were predicted to give equivalent selectivity (Fs < 3) to the one used throughout this study: Waters Atlantis dC18, Waters Atlantis T3 and Tosoh Bioscience TSKgel ODS-80Ts. These predictions, however, were not experimentally verified.

3.3.1.4. A word about ternary eluent composition. As stated previously in Section 3.2.1, the influence on the selectivity of the sample of the composition of organic modifier was also investigated by means of a 3D t_G–T–ternary resolution space (Cube C). It was found that AN yielded a larger robust region at lower analysis times than MeOH or mixtures of both, when used as the main component of eluent B, and was therefore selected for the final method.

3.3.2. Formal validation

A complete formal validation process was carried out for the selected method, the results which can be found in Appendix A. All acceptance criteria were successfully met indicating an accurate, precise, linear and rugged method.

3.4. Method control

A summary of the experimental parameter settings and tolerances for the final assay and purity indicating method of the eye drop sample are given in Table 4.

The final method has been designed with sufficient robustness that beyond good laboratory practices (equipment qualification/calibration, proper column maintenance, and general system suitability requirements) no critical factor need be tightly controlled in order to meet method performance criteria.

DryLab Robustness Module

Start | DryLab | Calculate | Robustness | Close | DryLab

Load | C:\Users\Kate\Desktop\Robustness paper\Robustness module calc\bot pH 27 AN.DLB

Outline

This module uses simulated data from previously created DryLab models to carry out robustness evaluations. Responses include critical resolution (Rs,crit) critical peak pairs (crit PP) and run time.

Method parameter		Tolerance		Units
gradient time (tG)	7	+/-	1	min
temperature (T)	25	+/-	2	°C
flow rate (F)	0.3	+/-	0.05	mL/min
start %B (S %B)	0	+/-	0.5	%
end %B (E %B)	100	+/-	0.5	%

Instructions

1. Click on "**Start**"to call up DryLab (the DryLab icon will appear on the task bar).
2. Click on "**Load**" to read in a DryLab *.dlb file.
3. Enter the method parameter settings and tolerances in their respective cells.
4. Click on "**Calculate**" to fill out the Results table.
5. Click on "**Close**" to close DryLab.

Note. Missing data in the Results table, arises from impossible experimental conditions such as Start %B < 0 or End %B > 100.

Experimental Conditions (DoE)[1]					
Exp.	tG	T	F	S %B	E %B
0	7	25	0.3	0	100
1	0	0	0	0	+
2	0	0	0	0	-
3	0	0	0	+	0
4	0	0	0	+	+
5	0	0	0	+	-
6	0	0	0	-	0
7	0	0	0	-	+
8	0	0	0	-	-
9	0	0	+	0	0
10	0	0	+	0	+
11	0	0	+	0	-
12	0	0	+	+	0
13	0	0	+	+	+
14	0	0	+	+	-
15	0	0	+	-	0
16	0	0	+	-	+
17	0	0	+	-	-
18	0	0	-	0	0
19	0	0	-	0	+
20	0	0	-	0	-

Results		
Rs,crit	crit PP	Run time[2]
2.14	7 / 8	7.7
2.15	7 / 8	7.8
2.14	7 / 8	7.7
2.15	7 / 8	7.8
2.43	7 / 8	7.4
2.44	7 / 8	7.4
2.44	7 / 8	7.4
2.45	7 / 8	7.4
1.81	7 / 8	8.2
1.82	7 / 8	8.2

Fig. 7. Software module used for multifactorial robustness calculations.

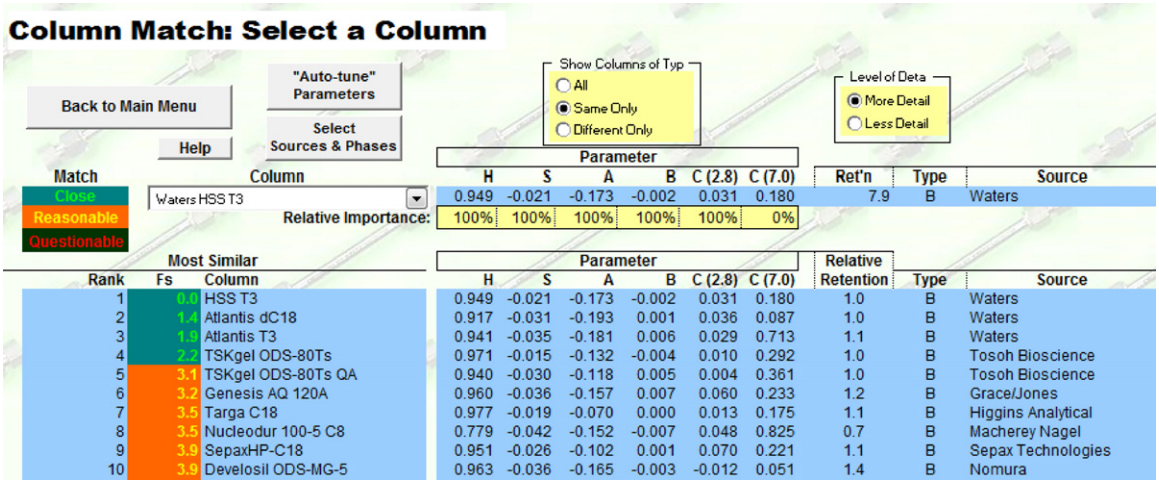


Fig. 8. Results from the ColumnMatch® database showing equivalent (Fs < 3) and similar (3 < Fs < 5) column selectivity to that of the column used in this study.

Table 4
Final assay and stability indicating impurity method.

Eluent A	50 mM KH_2PO_4 in water:AN (95:5, v/v)
Eluent B	50 mM KH_2PO_4 in water:AN (20:80, v/v)
pH of KH_2PO_4 solution	2.7 ± 0.1
Column	Waters HSS T3 C18 column (100 mm × 2.1 mm, 1.8 μm) *Potential substitution columns: Waters Atlantis dC18, Waters Atlantis T3 and Tosoh Bioscience TSKgel ODS-80Ts*
Gradient time	7 ± 1 min
Gradient shape	Start %B: 0 ± 0.5% End %B: 100 ± 0.5%
Column temperature	25 ± 2 °C
Flow	0.3 ± 0.05 mL/min
Critical peak pairs	1. (B) Imp-2, (A) Imp-4 2. (A) Imp-3, API-B
Detection	API-A and its impurities: 247 nm; API-B and its impurities: 297 nm
Run time	7 ± 1 min

4. Summary

A Quality by Design workflow was applied to the development of a fast, robust and reliable UHPLC method, with the assistance of the latest computer technologies. The method developed successfully passed through validation and has been used regularly and trouble free since that time. A robustness space was defined – providing an assurance of quality for the method – as a multidimensional space formed by tolerance windows of critically influential separation parameters (gradient time, temperature, pH, flow rate, start %B, end %B, stationary phase). The use of various modeling software programs allowed a limited amount of experimental data to be used to examine a large number of possible run conditions. Once the optimum run conditions and their tolerance to change were predicted, experiments were run to confirm the predictions. Thus the present approach maximized the information content of the initial experimental data while allowing a minimum number of final experimental runs to demonstrate robustness. This data in the future can on the one hand serve as a common medium of discussion between analysts and between analysts and overseeing regulatory bodies, and on the other help to easily and quickly diagnose any problems which may be encountered during the lifecycle of the method.

Acknowledgement

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Appendix A. Formal validation

Assay	*Procedure*	*Acceptance Criteria*	*Results*
Accuracy	Accuracy was determined by triplicate analysis of samples at three different concentration level (50, 100 and 150% level). The accuracy study was carried out for both active substances with two kinds of placebo at three concentration levels. Recovery was calculated at each concentration level for each point: $\text{Recovery}\,(\%) = \frac{(\%)}{(\%)_{calc}} \cdot 100$ (%) = Measured amount according to method description $(\%)_{calc}$ = Amount of API-A and API-B expressed as % of theoretical concentration	The relative standard deviation (RSD) for the individual recovery result at each level not more than 2.0%. The average recovery at different concentration levels: 98.0-102.0%.	Average recovery (%) and RSD (%) values at each level (50%, 100% and 150%) for API-A were 100.2, 100.6, 101.4 and 0.3, 0.4, 0.5, respectively. For API-B found values were 100.7, 101.1, 101.0 and 0.5, 0.1, 0.3. The accuracy of the analytical method was therefore found to be within the acceptance limits.
Linearity	The linearity ranges from 50 to 150% of the working concentration were studied. Two stock solutions containing API-A and API-B were prepared. Stock solutions were diluted to working concentrations and were injected once. Peak areas were plotted against concentration and regression line, Y intercept, slope and regression coefficient squared (R^2) were determined.	R^2 no less than 0.997 and Z value of no more than 3.0%. $Z = \frac{100 \cdot a}{b}$ a = Y intercept b= Area at 100% concentration level	R^2 and Z values for API-A were found to be 0.9993 and -1.1, respectively. For API-B these values were found to be 0.9995 and 0.1, respectively. Therefore, satisfactory linear relationship between the peak areas and the solution concentrations across the evaluated range were verified.
Precision	Six independent preparations of 1.0 ml of eye drop solution API-A 0.2% API-B 0.5% diluted by a factor 50 were tested. For the investigation of intermediate precision the method precision test was repeated by another analyst on a different instrument, starting from preparing eluent, standard and sample solutions.	The RSD for six preparations for each analyst not more than 2.0%. The relative difference between the two analysts not more than 3.0%. For both active substances a relative difference in the average results not more than 3.0%.	The RSD for each analyst measuring API-A and API-B did not exceed 0.2% and the maximum relative difference found was 0.3%. Therefore the precision of the analytical method was found to be within the acceptance limits.

<table>
<tr><th>Purity</th><th>Procedure</th><th>Acceptance Criteria</th><th>Results</th></tr>
<tr><td>Accuracy</td><td>Accuracy was determined by triplicate analysis of samples at four different concentration level (from reporting level – 0.1% - to the 120% of the specification limit).
$$\text{Recovery (\%)} = \frac{(\%)}{(\%)_{calc} + (\%)_{unsp}} \cdot 100$$
(%) = Amount of impurity found in spiked sample
$(\%)_{calc}$ = Amount of added impurity (100 % equivalent to 0.04 mg /mL API-A and 0.1 mg/mL API-B)
$(\%)_{unsp}$ = Amount of impurity found in unspiked sample</td><td>The average recovery on different concentration levels:<table><tr><td>Concentration level</td><td>Average recovery (%)</td></tr><tr><td>RL (≥QL) - 0.05%</td><td>80-120%</td></tr><tr><td>0.05 – 0.50%</td><td>85-115%</td></tr><tr><td>0.51 – 2.0%</td><td>90-110%</td></tr><tr><td>2.1 – 10.0%</td><td>95-105%</td></tr></table></td><td>The accuracy of the analytical method was verified to be within the acceptance limit.</td></tr>
<tr><td>Sensitivity</td><td>The quantitation limit (QL) and detection limit (DL) concentrations were determined based on the signal to noise ratio (S/N). The prepared solutions at detection and quantitation level were injected.</td><td>The quantitation limit for all components not more than the reporting limit (0.1%). S/N at detection level 2:1 or 3:1. S/N at quantitation level 10:1. The relative standard deviation of six injections at quantitation level not more than 20%.</td><td>S/N is approximately 3 at detection level and 10 at quantitation level. Suitable RSD could be achieved at quantitation level and this level is under the reporting level in each case.</td></tr>
<tr><td>Linearity</td><td>Five solutions are studied in the range from quantitation limit (QL) to 120% of the specification limit by dilution of two stock solutions.</td><td>The regression coefficient squared (R^2) not less than 0.990.</td><td>A satisfactory linear relationship between the peak areas and the solution concentrations across the range defined was found.</td></tr>
<tr><td>Precision</td><td>Six independent preparations of 1.0 ml of eye drop solution API-A 0.2% API-B 0.5% diluted by a factor 50 were tested. Six drug product vials/bags were tested and one injection from each vial/bag for each strength was performed. Each sample was measured once and the relative standard deviation of the individual impurity results for each strength was calculated. For the investigation of intermediate precision the method precision test was repeated by another analyst on a different instrument, starting from preparing eluent, standard and sample solutions.</td><td>The relative standard deviation for six preparations on different concentration level for each analyst:<table><tr><td>Concentration level</td><td>RSD (%)</td></tr><tr><td>RL (≥QL) – 0.50%</td><td>NMT 20%</td></tr><tr><td>0.51 – 2.00%</td><td>NMT 15%</td></tr><tr><td>2.10 – 10.00%</td><td>NMT 5%</td></tr></table>The relative difference between the two analysts on different concentration level:<table><tr><td>Concentration level</td><td>Relative difference (%)</td></tr><tr><td>RL (≥QL) – 0.10%</td><td>NMT 60%</td></tr><tr><td>0.11 – 0.25%</td><td>NMT 40%</td></tr><tr><td>0.26 – 1.0%</td><td>NMT 25%</td></tr><tr><td>1.1 – 10.0%</td><td>NMT 15%</td></tr></table></td><td>The precision of the analytical method for the unstressed sample was not calculated because all impurities are below reporting limit (<0.1%). The precision of the analytical method for the stressed sample is within the acceptance limit.</td></tr>
</table>

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