**In silico** robustness testing of a compendial HPLC purity method by using of a multidimensional design space build by chromatography modeling—Case study pramipexole

Alexander H. Schmidt\(^a,b,\ast\), Mijo Stanic\(^a\), Imre Molnár\(^c\)

\(^a\) Steiner & Co., Deutsche Arzneimittel GmbH & Co. KG, Wallenroder Strasse 12-14, 13435 Berlin, Germany  
\(^b\) Freie Universität Berlin, Institute of Pharmacy, Königin-Luise-Strasse 2+4, 14195 Berlin, Germany  
\(^c\) Molnár-Institute, Schnegglöckchenstrasse 47, 10407 Berlin, Germany

**A R T I C L E   I N F O**

Article history:
Received 14 September 2013  
Received in revised form 18 December 2013  
Accepted 21 December 2013  
Available online 31 December 2013

Keywords:
HPLC method development  
Quality by Design  
Design Space  
Robustness in routine quality control  
Pramipexole

**A B S T R A C T**

Purity testing of the active pharmaceutical ingredient (API) pramipexole is performed using an official (compendial) and harmonized method published in the European Pharmacopeia (E.P.) and United States Pharmacopeia (USP). According to this monograph the successful chromatographic separation of the API from impurities is achieved on a C18 column with gradient elution of an ion pairing buffer of pH 3.0 (mobile phase A) and acetonitrile (mobile phase B).

Although not recommended in general, compendial methods are often adapted for purity testing of generic formulations. In this paper a novel approach to evaluate method robustness of an adapted method – prior of full method validation – is described. Based on Quality-by-Design (QbD) principles, a small number of experiments are performed, which after entering them into a chromatography modeling software allow to visualize a multidimensional “Design Space”, a region, in which changes in method parameters will not significantly affect the results as defined in the ICH guideline Q8(R2) leading to a more flexible method handling in routine analysis.

For two different recommended C18 columns a multidimensional Design Space (Method Operating Design Region, MODR) was constructed to study the robustness of the adapted method with a newly developed Robustness Module. In a full factorial design the following six parameters were varied at three levels (low, nominal, high): gradient time, temperature, pH of the aqueous eluent (A), flow rate, start- and end concentration of the organic mobile phase component (eluent B). The resulting 3\(^6\) = 729 experiments were performed in silico from the previously constructed models for Design Space in less than 1 min and showed that the required resolution of 2.0 could not be reached in all experiments for the two columns which were recommended by the E.P. (failure rate 25% and 16%, respectively). However, by adjusting the gradient time, we were able to fulfill the requirements with a failure rate of zero.

For the aqueous eluent a separate “Eluent Design Space” study was performed, which allows the construction of ionic strength vs. ion pairing concentration models to identify the optimum combination of the concentrations for the buffer and the ion-pairing reagent.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

In an increasingly globalized world, the development of pharmaceutical products and their manufacturing are often in different companies and in different parts of the world. In a registration dossier – called Common Technical Document (CTD) – all necessary information about the chemistry, the manufacturing and the controls (CMC) of the drug are presented in compliance to the ICH guideline M4Q(R1)\(^1\) and approved or rejected by regulatory agencies (FDA, EMA, etc.).

In the section 3.2.P of the CTD the pharmaceutical and analytical development should be described, e.g., the development of liquid chromatographic methods for purity testing, assay and content uniformity. The common method development strategies in liquid chromatography range from an obsolete trial-and-error approach, for example by varying one-factor-at-a-time (OFAT), to more systematic ways, for example by using software modeling packages (e.g. DryLab\(^\circledR\) 4, ChromSword, ACD/LC simulator)\(^2–9\).

Nowadays, some Quality-by-Design approaches use more statistical concepts with experimental design plans as an efficient and...
Fast tool for method development. Quality-by-Design (QbD) is a concept outlined years ago by Juran [10] but only recently applied in the pharmaceutical industry. It is defined by the ICH guideline Q8(R2) [11] as “a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management”. And further on the Design-of-Experiments (DoE) is defined as “A structured, organized method for determining the relationship between factors affecting a process and the output of the process” [11].

A key component in the development of analytical procedures using QbD is targeting the Design Space (DS) [12]. The ICH guideline Q8(R2) describes the DS as “The multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality. Working within the design space is not considered as a change. Movement out of the design space is considered to be a change and would normally initiate a regulatory post approval change process. Design space is proposed by the applicant and is subject to regulatory assessment and approval” [11].

In case of generic drugs, it is often a common practice to adapt Pharmacopeia compendial methods (e.g., for assay and purity) from the monograph of the corresponding active pharmaceutical ingredients. Although the adaption of compendial methods cannot be recommended in general, especially for older methods which are not state-of-the-art and which should be updated [13], it is often an easy strategy especially, when there is lack of experience in method development.

To demonstrate the suitability of a compendial method under actual conditions of use (product-specific release testing) the method has to be validated in compliance with regulatory requirements (e.g. ICH guideline Q2(R1) [14]). The requirements usually are that the method has to be specific, precise, accurate and robust. The ICH guideline Q2(R1) define “the robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal use” [14].

Our experience as a contract lab for release testing of drugs indicates, these robustness tests were often not performed due to their
complexity to the many possible experimental designs and lack of guidance [15]. Therefore, the aim of our study was to demonstrate the potential of chromatography modeling – in an easy way – to evaluate the robustness of an already existing (e.g. adapted compendial) method.

In addition, our concept proofs that robustness testing is not time-consuming at all and should be applied parallel to statistical methods side by side.

With a limited number of experiments around the conditions of the existing method, the modeling software constructs a multi-factorial Design Space (also known as the Method Operating Design Region, MODR). After verification, the use of the Design Space can be extended to evaluate the robustness of the method. Therefore, our innovative approach uses the recently implemented Robustness Module of a method modeling software to perform in silico robustness testing in a full factorial design within a few seconds. The potential of this approach will be demonstrated on the compendial purity method for pramipexole.

The S-enantiomer of pramipexole – the IUPAC name is (S)-2-amino-6-N-(propylamino)-4,5,6,7-tetrahydrobenzo thiazole – is indicated as a symptomatic treatment for Parkinson’s disease and restless legs syndrome [16]. The salt form commonly used as the active pharmaceutical ingredient in solid pharmaceutical formulations (tablets, extended-release tablets) is dihydrochlorid monohydrate.

Purity testing of the active pharmaceutical ingredient (API) pramipexole is performed using an official (compendial) and method described in the monograph of the European Pharmacopeia (E.P.) [17] and the United States Pharmacopeia (USP) [18]. According to this monograph High-Performance Liquid Chromatography achieves the successful chromatographic separation of the API from impurities on a C18 column with gradient elution of an ion-pairing buffer of pH 3.0 and acetonitrile as the organic mobile phase. All chromatographic parameters of the E.P. and USP monographs are identical, except the column dimensions (E.P.: 125 mm × 4.6 mm, 5 μm particle size vs. USP: 150 mm × 4.6 mm, 5 μm particle size). Typically no information about column brands are given in official monographs but suitable columns can be found in the EDQM knowledge database [19]. The monograph requires for testing of the impurities A, B, C and E (see Fig. 1), while impurity D is the chiral isomer and separated by a different method.

2. Experimental

2.1. Chemicals and eluents

Acetonitrile was HPLC-gradient grade, all other chemicals were at least analytical grade and purchased from Merck (Darmstadt, Germany). Water used was purified by a TKA water purification system (Thermo Fisher Scientific, Dreieich, Germany).

2.2. HPLC equipment

Chromatographic runs were made on an ACQUITY UPLC® H-class system, which can be used in an UHPLC as well as in an HPLC mode. It consists of a Quaternary Solvent Manager with Solvent Selection Valve, Sample Manager, Column Controller, and Photo-Diode Array detector with Empower® 2 C/S-software (Waters, Eschborn, Germany). The setup was used to acquire, store and process the chromatographic raw data. The UV detection of the compounds of interest was carried out at 264 nm and the UV spectra were taken in the range of 200–400 nm. The spectral data were used by the purity function of the Empower software to confirm that all peaks are spectrally pure or that co-elution of components occurs.

2.3. Chromatographic conditions

The knowledge database of the European Pharmacopeia recommended Symmetry C18 (Waters, Eschborn, Germany) or Inertsil ODS-2 (GL Sciences, Tokyo, Japan) columns with the dimension 125 mm × 4.6 mm and 5 μm particle size as suitable stationary phases to separate the impurities A, B, C and E from pramipexole [19]. Since this column length is commercially not available from both companies, the 150 mm × 4.6 mm columns were used, as recommended by the USP [18].

The compendial monograph describes a gradient separation applying a linear gradient from 20 to 40% eluent B in 15 min. Eluent A was a buffer, prepared by dissolving 9.1 g of potassium dihydrogen phosphate (corresponds to 67 mM) and 5 g of sodium octanesulfonate monohydrate (corresponds to 21 mM) in 1000 ml water. The pH of 3 was adjusted with phosphoric acid. Eluent B was acetonitrile. The flow-rate was set to 1.5 ml/min, column temperature was 40 °C and the injection volume was 5 μl [17,18]. Usually the flow-rate has to be adjusted in compliance to chapter 2.2.46 of the European Pharmacopeia when column dimensions are changed. Due to the fact that the USP recommends a 150 mm × 4.6 mm column no adjustments were made.

2.4. Software for chromatography modeling

DryLab®4 chromatography modeling software package (Molnár-Institute, Berlin, Germany), which includes PeakMatch and the 3-D-Robustness modules, was used for chromatography modeling. The retention times and peak areas of individual peaks from the experimental runs were used as input data for chromatography modeling [20].

Fig. 2. Design of Experiments to build a three-dimensional Design Space (a) and a separate two-dimensional Eluent Design Space (b) for each of the two columns.
Fig. 3. (a) 3D Design Space and the corresponding 2D resolution map at pH 3.0 for the Inertsil ODS-2 column. (b) Frequency of the distribution of the resolution values $R_{s,crit}$ for all 729 experiments of the robustness study on the Inertsil ODS-2 column.
Fig. 4. (a) 3D Design Space and the corresponding 2D resolution map at pH 3.0 for the Symmetry C18 column. (b) Frequency of the distribution of the resolution values $R_{\text{crit}}$ for all 729 experiments of the robustness study on the Symmetry C18 column.
2.5. Preparation of system suitability standard solution

The content of one vial "Pramipexole for system suitability CRS" (15 mg, available from EDQM, Strasbourg, France) is solved in about 2 ml diluent, transferred into a 10 ml volumetric flask and filled up to the mark with diluent. A mixture of acetonitrile and buffer (20:80) was used as the diluent.

This system suitability standard solution contains pramipexole, the impurities A, B, C and E as well as two unknown impurities.

2.6. Design of Experiments (DoE) for modeling

Initial input data for each of the two suitable columns were acquired in a way, that the parameters of the compendial method were at the center point and a set of 12 experiments around that point were performed under the following conditions: Gradient times: tG1: 8 min and tG2: 24 min, temperatures: T1: 27°C and T2: 54°C. The pH-values of the buffer were pH1: 2.7, pH2: 3.0 and pH3: 3.3.

Since the eluent A contained – in addition to the buffer – an ion pairing reagent, an independent eluent study for each of the two columns was performed: a set of 9 experiments with different ionic strength and ion pairing concentrations around the center point (concentrations of the official method) were performed with the following values: ionic strength (concentrations of potassium dihydrogen phosphate) Ck1: 33 mM, Ck2: 67 mM and Ck3: 100 mM. Concentrations of the ion pairing reagent (sodium octanesulfonate monohydrate) Cipr1: 11 mM, Cipr2: 21 mM and Cipr3: 32 mM. All other parameters were as described in the monograph of pramipexole.

3. Results and discussion

3.1. Development strategy

In order to define a Design Space for the adapted compendial HPLC purity method, we follow Quality-by-Design (QbD) principles [13,21,22], which can be divided into the five steps (1) definition of method goals, (2) risk assessment, (3) Design of Experiments, (4) Design Space building and robustness testing followed by (5) method control strategy based on the knowledge gained about the method.

3.1.1. Step 1 – definition of method goals

The primary goal of method development of an HPLC purity method is generally to separate the API from impurities that may impact the quality of the pharmaceutical formulation (with resolution Rs, crit > 2.0). In this case we studied the robustness of an official method for purity testing of pramipexole (API), which was proposed to be adapted for purity testing in pharmaceutical development of a generic formulation. Essential part of this Quality-by-Design approach was to create a visual multi-factorial "Design Space", in which the robustness of the method can be studied. In addition, a separate "Eluent Design Space" should identify if the combination of concentrations for the buffer and the ion pairing reagent are at its optimum.

3.1.2. Step 2 – risk assessment

Also an important part of this QbD approach is an early risk assessment to identify the critical parameters, which could affect method performance [23]. That could be method factors, which...
Fig. 5. Frequency distribution of the critical resolution values $R_{s,\text{crit}}$ for all 729 experiments of the robustness study after adjustments of the gradient time (a) Inertsil ODS-2 column (b) Symmetry C18 column.

may affect extraction of the compounds of interest from the pharmaceutical formulation (e.g. extraction method, time, and solvent) [24] and will not further explained here, as well as settings of the HPLC system. The high-risk influential separation parameters stationary phase, gradient time $t_G$, temperature $T$, and pH of the eluent A were identified [25] and assessed experimentally using a Design-of-Experiments (DoE) methodology.

Only a reduced number of separations are highly influenced by the ionic strength of the eluent and/or ion pairing reagent concentrations [26]. But because the method for pramipexole in the E.P. uses both, buffer and an ion pairing reagent in the aqueous eluent, a separate “Eluent Design Space” should be established to identify the best combination of concentrations for the buffer and the ion pairing reagent.
3.1.3. Step 3 – Design of Experiments

From previous works we know the advantages of two-dimensional [27] and three-dimensional resolution maps [13] with the choice to calculate the influence of additional 7–8 factors, very well. The goal of this work was to establish a multi-factorial Design Space and use it for in silico robustness testing by using the recently introduced Robustness Module of the DryLab®4 software. Our goal was to increase the flexibility of the method handling in the routine work and reduce the time of the process development from months to days.

Therefore the experimental data from 12 experiments for each of the two columns were entered into the software, which then simulates and predicts separations for a very large number (>$10^6$) of variations in chromatographic conditions.

Initial input data were acquired under the conditions mentioned before. The set of twelve (4 × 3) experiments were performed on each of the two columns according to the Design of Experiments are shown in Fig. 2a [28].

In the same way the independent “Eluent Design Space” study was conducted, in which the combined influence of the ionic strength vs. the ion pairing reagent concentration was evaluated. A set of nine experiments were performed on each of the two columns in which the concentration of the buffer and the ion pairing reagent were varied, according to the Design of Experiments in Fig. 2b. All other chromatographic parameters were kept constant at the conditions mentioned in the monograph.

3.1.4. Step 4a – building of a multi-factorial Design Space

The retention times and areas of the peaks of pramipexole, impurities A, B, C, and E and the unknown impurities 1 and 2 were matched in each of the chromatograms by using the Peak-Match module of the DryLab®4 software [13,20]. In this process peak tracking was confirmed by peak area and the UV-spectra of the compounds. The experimental data of the chromatograms were necessary in order to build 2D-models and use them for the further calculation of 3D-resolution cubes for each of the 2 columns, in which the combined influence of the chromatographic parameters are visualized and can be studied (see Figs. 3a and 4a). The color code in these resolution maps represents the value of the critical resolution, with warm “red” colors showing large resolution values ($R_c > 2.0$) and cold “blue” colors showing low resolution values ($R_c < 0.5$) [8]. The visual inspection of the cube can easily be done, by resetting the resolution option so that the Design Space is isolated and is shown in form of irregular geometric bodies. A large red
region indicates where the method is very robust and the resolutions of all peaks in the chromatogram are well baseline separated from each other ($R > 2.0$).

It can be seen, that the adapted method on the recommended Inertsil ODS-2 (Fig. 3a) and Symmetry C18 (Fig. 4a) columns are very close to the edges of failure when the gradient time $tG$ is slightly increased. It is highly probable that the method on these two columns will fail in robustness testing, as calculations of the DryLab Robustness Module approved (see further ahead).

Adjustments of the gradient time from the recommended 15 min to shorter run times, e.g. 10 min, would not even reduce the analysis time but also would shift the working point into the geometric center point of the Design Space, where the method is robust.

To confirm the multi-factorial Design Space on the Inertsil ODS-2 and Symmetry C18 columns, a comparison of predicted and experimental retention times for the adjusted working point and six verification points around the adjusted working point within the design space was performed. In Table 1 it can be seen that the predicted retention times for all compounds were found to in excellent agreement to the experimentally observed ones since the average deviations were less than 1.7%. In addition the prediction of the critical resolution was also evaluated. As summarized in Table 1 the deviation between the predicted and experimentally observed critical resolution were between 1.09 and 2.77%.

The high correlation between DryLab predicted and experimental data is in agreement with previous reported data (see also Fig. 7 further ahead) [13,26,29–31].

3.1.5. Step 4b – robustness testing

After the Design Space was confirmed, it can be used to investigate the robustness of the method prior to method validation. Robustness studies typically utilize full or fractional factorial designs to estimate the effect of variabilities in individual method parameters and their interaction with each other [18]. In this paper, the outcome of the robustness study for the adapted method was studied with the aid of the Robustness Module of the DryLab®4 software without the need for further experiments. This module uses the constructed 3D resolution cubes for each of the two columns for multi-factorial robustness calculations. The following six experimental factors were varied at three levels (high, nominal and low value).

Gradient time $tG$ (15 min ± 1 min), temperature $T$ (40 °C ± 4 °C), pH (3.0 ± 0.3), flow rate (1.5 ml/min ± 0.15 ml/min) and the %start (20% ± 2%) and %end (40% ± 2%) of the eluent B composition of the gradient.

![Chromatogram](image)

**Fig. 7.** Predicted chromatogram (a) and experimental chromatogram (b) of pramipexole for system suitability CRS.
These tolerances are much larger than the specification for precision for HPLC systems and allow an evaluation of the robustness of the method, especially when transferred into other labs and performed on different equipment.

The results of the 729 experiments (full factorial design for 6 parameters at 3 levels) are calculated by DryLab® in less than 1 min. In comparison to other software packages, this novel Robustness Module can not only summarize the results of the robustness study in a table or graph but also a predicted chromatogram for every simulated experiment is available.

From the results it can be seen that the required resolution of $R_{S, crit} = 2.0$ cannot be reached in all experiments for the Inertsil ODS-2 and Symmetry C18 columns (failure rate 25% and 16%, respectively; see also Fig. 3b and 4b). However, after adjustment of the gradient time to $T_G = 10$ min, the number of experiments on the Inertsil ODS-2 and Symmetry C18 columns, that would be outside of the required resolution range ($R_{S, crit}$) is zero (failure rate 0%, see also Fig. 5a and b). That means, that practical all experiments fulfill the critical resolution requirements, which indicated, that the method could be considered robust.

The frequency distribution for each of the two columns show how often (N) a certain critical resolution $R_{S, crit}$ occurs under combination of possible, true parameter tolerance values.

3.1.6. Step 4c – Eluent Design Space study

By using the modeling software DryLab®2010, the influence of the ion strength vs. ion pairing reagent concentration can be studied for each of the two columns. Therefore the experimental data of the nine chromatograms of the separate “Eluent Design Space” study were used to establish 2D-models (see Fig. 6a and b). The color code in these resolution maps is the same as mentioned above and represent the value of the critical resolution.

From these 2D resolution maps of both columns it can be seen that using the concentrations of the buffer and the ion pairing reagent recommended in the official monograph (67 mM and 21 mM, respectively), are once again close to the edges of failure, where the method is not robust and peak co-elution is possible. Adjustments by reducing the concentrations to 55 mM buffer and to 14 mM of the ion pairing reagent would result in a more stable and therefore robust method.

As an example, a DryLab predicted and experimental chromatogram at the adjusted working point on a Symmetry C18 column is shown in Fig. 7a and b.

3.1.7. Step 5 – method control strategy

A method control strategy as recommended by the ICH Q8 guideline was implemented as the last step of the Quality-by-Design workflow to ensure, that the method is performing as intended on a routine basis. Based on the robustness of the adjusted method on the Inertsil ODS-2 or Symmetry C18 columns, a system suitability test should be the only one control element needed [13].

The critical resolution for each of the peak pairs of the pramipexole for system suitability CRS, which contains pramipexole, the impurities A, B, C and E, as well as two unknown impurities, was chosen as a system suitability test parameter and should not be lower than 2.0 ($R_{S, crit} > 2.0$).

The method is currently applied for purity testing in the pharmaceutical development of different pramipexole containing generic formulations and can also be used for assay and content uniformity testing of the drug product. It has to be validated once the final composition of the developed generic formulation is fixed. But because of the knowledge gained of the method, unexpected results in the validation process are nearly impossible.

3.1.8. Step 6 – continual improvement

Currently, we are planning to repeat the basic experiments of the DryLab models, to try out better columns and eluents to further adjust or improve the position of the working point.

4. Conclusion

In this paper Quality-by-Design principles were used for a common strategy of adapting an official (compendial) purity method instead of developing a new one. Therefore, a Design Space – a volume in which the method is robust – is defined and visualized by using a chromatography modeling software. Using the recently introduced Robustness Module, robustness testing can be performed in silico. It resulted, that the initial method recommended by the European Pharmacopoeia on Inertsil ODS-2 or Symmetry C18 columns was not very robust against variation of the gradient time. After reducing the gradient time from 15 to 10 min, the adjusted method is very robust and shows a failure rate of 0% in our study.

A separate Eluent Design Space study was performed to identify the optimum concentrations for the ionic strength (55 mM of the buffer) and the ion pairing reagent (14 mM), which were lower than the concentration mentioned in the monograph (67 mM and 21 mM, respectively).

Besides of the importance to performed a robustness study prior of method validation to be prepared for unexpected surprises with the performance of the method – especially when adapting a method over developing a new one is chosen – this innovative concept of in silico robustness testing is a contribution to green chemistry by reducing mobile phase waste through computer modeling instead of excessive testing.

References


