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UHPLC method for multiproduct pharmaceutical analysis by Quality-by-Design



Ludivine Ferey^{a,*}, Adrien Raimbault^a, Isabelle Rivals^b, Karen Gaudin^a

^a ChemBioPharm Team, ARNA Laboratory, INSERM U1212, CNRS UMR 5320, Bordeaux University, France

^b Equipe de Statistique Appliquée, ESPCI Paris, PSL Research University, UMRS 1158, 75005 Paris, France

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ABSTRACT

An innovative Analytical Quality-by-Design (AQbD) methodology was followed to develop a specific and robust UHPLC method for the simultaneous separation of 16 active pharmaceutical ingredients (APIs). In the context of pharmaceutical repositioning, these molecules have been selected as good candidates for buccal per mucous (BPM[®]) administration route. Given the structural and physico-chemical diversity of compounds, an innovative development strategy based on QbD was applied. The main advantage of QbD is to ensure the robustness of the method. During a first scouting phase, the C18 chromatographic column was selected. Throughout the study, acetonitrile and ethanol based-mobile phases were investigated and compared. Ethanol was chosen as an alternative to acetonitrile due to its green properties coming from its lower toxicity and sourcing from renewable sources. Screening designs were performed to identify critical process parameters (CPPs). In ethanol media, temperature turned out to be a critical factor on peak retention and separation. Response surface methodology was then carried out to optimize CPPs and define the experimental domain of the method where complete separation between all peaks was obtained. Because changes in the elution order of the compounds occurred when modifying the experimental conditions, time differences between peaks were chosen as critical quality attributes, and an original data treatment was developed. It consisted in a systematic modelling of the time intervals between all possible pairs of peaks over the whole 3D experimental domain. Finally, a desirability analysis based on the smallest predicted time interval between peaks enabled to find optimal conditions only with ethanol based-mobile phases. Optimal conditions using ethanol, a Xbridge BEH Shield RP18 column and a 500 mL starting isocratic step, were determined by maximizing the desirability value and corresponded to a gradient slope of 2.57 %/min, a pH of 4.85, and a temperature of 33.7 °C. A baseline separation of the 16 APIs was achieved with resolutions superior to 2.4 and the robustness of the method was experimentally validated.

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1. Introduction

This study takes place in the context of drug reformulation and repositioning in pharmaceutical industry. The innovation is based on the new per-mucous gingivo-jugal administration route (Buccal per Mucous route, BPM[®] [1,2]), which offers instantaneous systemic bioavailability and fast pharmacodynamic efficacy at very low useful dosages compared to the other usual injectable formulations, as already clinically demonstrated [3,4]. The pharmaceutical formulation is a hydro-alcoholic solution containing 40% to 65%

ethanol by mass. Based on literature, 16 active principal ingredients (APIs) were identified as candidates for this new innovative and non-invasive route of administration: doxylamine and melatonin for sleep disorders, midazolam for treating episodes of epilepsy and convulsions, ondansetron, domperidone, and metopimazine as anti-emetic treatments in oncology, 5 statins for the treatment of hypercholesterolaemia and the prevention of myocardial infarction, quinine and artemether to treat malaria, diclofenac and ketoprofen as nonsteroidal anti-inflammatory drugs, and paracetamol, an analgesic. Their chemical structures, molecular weights (MW), pKa, and logP values are presented in Fig. 1.

A pre-formulation step of such pharmaceutical development consists in a solubility study of each molecule candidate in hydro-alcoholic solutions in order to assess its potential for BPM[®] new delivery route. Indeed, highly concentrated fluids are targeted to

* Corresponding author at: ChemBioPharm Team, ARNA Laboratory, INSERM U1212, CNRS UMR 5320, Bordeaux University, 146 rue Léo Saignat, 33076 Bordeaux Cedex France.

E-mail address: ludivine.ferey@u-bordeaux.fr (L. Ferey).

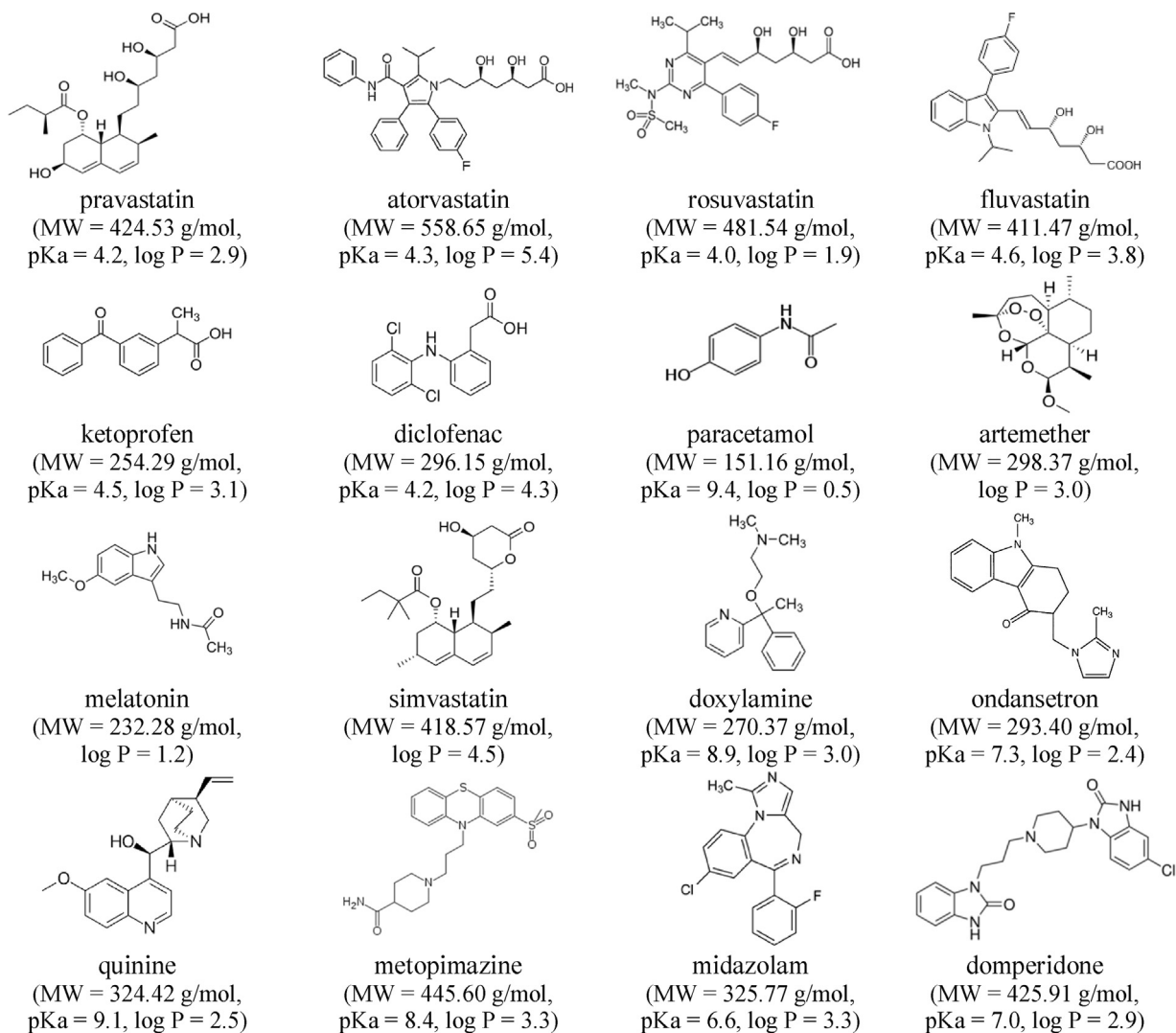


Fig. 1. Chemical structure, molecular weight, pKa, and logP (at 25 °C) values of the 16 investigated drugs.

deliver the adequate dosage of API through BPM[®] administration route. To support formulation development and quality control activities of each API in BPM[®] formulations, a chromatographic method was required. Instead of developing one method for each compound [4] or for compounds of a same family, e.g. statins [5], a unique method was targeted in this study. In fact, generic methods can prove to be very useful in industry for multiproduct quality control activities.

The UHPLC-UV method should allow the simultaneous separation of the 16 active molecules, so that only one method will be required for the 16 APIs, saving time while ensuring molecule identity through method specificity. In the framework of an industrial partnership in a pharmaceutical environment and given the complexity of the mixture to be separated, an Analytical Quality-by-Design (AQbD) approach was followed. Since the implementation of ICH pharmaceutical development guidelines, Q8(R2) [6], QbD is increasingly applied to the development of separation methods in pharmaceutical analysis [7–13]. One of the most important pharmaceutical applications of AQbD is multiproduct analysis, i.e. the separation of several APIs, for detection of counterfeit medicines [14–19] or for homologous molecule analysis in quality control [20].

QbD strategy is based on multivariate techniques allowing a faster method development process and a deeper method knowl-

edge. Indeed, QbD uses design of experiments (DOE) which is an efficient tool to investigate simultaneously a high number of experimental factors in a limited number of trials. The main goal of the QbD methodology is to simultaneously optimize the separation and estimate method robustness, through the determination of a Design Space (DS). In ICH Q8(R2), the DS is defined 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” [6]. Thus, the DS corresponds to the subspace of the experimental domain where method quality is guaranteed. As stated by ICH Q8(R2) guideline [6]: “working within the design space is not considered as a change”, recognizing the DS as a zone of robustness of the chromatographic method where specific quality criteria are met. Therefore, quality criteria, called critical quality attributes (CQAs), were selected to assess method performances. Considering chromatographic methods, CQAs are characteristic responses of chromatograms that should be within an appropriate limit or range to ensure the quality of the separation. CQAs are chosen in accordance with the objectives of the method stated in the Analytical Target Profile (ATP) [12,21]. In chromatography, mainly resolutions (*R*_s) between adjacent peaks have been used as CQAs [10,16,22,23]. However, when dealing with complex mixtures of structurally unrelated compounds for which inversions of elution

order are observed over the 3D space, Rs cannot be used as CQAs since they are no longer monotone functions and therefore cannot be well modelled with multiple linear regressions. This is why Lebrun et al. [24] proposed the use of the separation criterion (S) defined as the time difference between the beginning of the second peak ($t_{B,2}$) and the end of the first peak ($t_{E,1}$) of two consecutive peaks ($S = t_{B,2} - t_{E,1}$; with $t_{B,2} > t_{E,1}$) [14,15,17,19]. In this study, the S criterion was retained as CQA to circumvent computational difficulties resulting from the changes in elution order likely to occur while varying experimental conditions.

First, scouting experiments were carried out to select the most adequate C18 stationary phase and organic solvent. Due to its green properties, ethanol was selected as an alternative organic solvent to acetonitrile. Moreover, as the purpose of this development was to provide a method for API analysis in ethanol/water mixtures, the use of ethanol as green chemistry approach appeared relevant. Ethanol can afford eluotropic strength similar to acetonitrile but the main drawbacks of ethanol compared to acetonitrile is its high viscosity and UV cut-off [25–27]. A comparison study of these two organic solvents was carried out and critical process parameters (CPPs) on retention times and separation were identified by means of screening designs. An original optimization approach was then followed, based on the systematic computation of the time intervals between all the possible pairs of peaks over the whole 3D experimental domain [28]. The optimal conditions were predicted by applying a desirability analysis on the smallest time interval between two consecutive peaks, finally leading to the establishment of an experimental domain where complete separation of all peaks was obtained. The overall objective of the present work was to demonstrate the ability of such innovative optimization strategy based on QbD to develop specific and robust UHPLC methods for multiproduct analysis.

2. Material and methods

2.1. Chemicals and reagents

HPLC-grade acetonitrile (ACN), HPLC-grade ethanol (EtOH) 96%, ammonium acetate (99%), sodium bicarbonate ($\geq 99.7\%$), formic acid (99%), and acetic acid ($\geq 99.9\%$) were purchased from VWR chemicals (VWR International, Fontenay-sous-Bois, France). Ammonium formate ($\geq 99.995\%$) was supplied from Sigma Aldrich (St Louis, MO, USA). HPLC grade water (minimum resistivity of 18.2 M Ω) was produced in house by ELGA Milli-pore system (France).

2.2. Sample and buffer preparation

The structures of the 16 APIs with their physico-chemical properties are reported in Fig. 1. All these compounds were provided by JKphArma (JKphArma Galenic Innovation SAS, Cenon, France).

Stock solutions of individual molecules were prepared at concentrations of 1 mg/mL or 5 mg/mL (except artemether stock solution prepared at 10 mg/mL) in water and/or EtOH 96%, depending on solubility properties.

To identify peaks and save time during method development, several working mixtures composed of 4 or 5 molecules were injected. Spectra information offered by UV diode array detector also allowed an easier peak assignment. Mixtures were prepared by mixing appropriate volumes of stock solutions and diluting in water-ethanol 96% (50:50, v/v) to achieve a concentration of 0.125 mg/mL for most molecules, 0.025 mg/mL for paracetamol, quinine, domperidone, and midazolam, and 2 mg/mL for artemether to be able to detect it at 210 nm. Concentrations of compounds in working mixtures were adjusted to obtain similar peak heights.

Formate and acetate buffers were prepared from 20 mM ammonium salt solutions and pH was adjusted to the desired value (2.6, 3.8, and 5.6) with formic or acetic acid, respectively, using a Radiometer Analytical pH meter (Hach, France). For pH values of 7.4 and 8.6, buffers were prepared from a 10 mM sodium bicarbonate solution, pH being adjusted to the desired value with formic acid.

During gradient analysis, the ionic composition of the mobile phase was kept the same.

2.3. Instrumentation

Chromatographic experiments were performed on an Accela UPLC system (Thermo Scientific, USA) equipped with a pumping device, an autosampler, a column oven, and a diode array detector. The quaternary solvent delivery pump was able to work up to pressure of 1000 bar. The autosampler holds a 25 μ L standard loop. The injected volume was fixed at 5 μ L. Chromatograms were systematically recorded in UV from 200 to 400 nm with a resolution of 1.0 nm and an acquisition rate of 10 Hz. Peak integrations were performed at 210 nm, 254 nm, and 278 nm as the best compromise in terms of detectability for the studied drugs.

The chromatographic columns used in this study were: an Acquity UPLC BEH C18 (hybrid, trifunctional C18, fully end-capped), a Xbridge BEH Shield RP18 (hybrid, monofunctional C18, embedded polar group, fully end-capped), and a Cortecs C18+ (solid-core particles, trifunctional C18, positively charged surface, fully end-capped) with the same dimensions of 100 mm x 3.0 mm I.D. for both Acquity UPLC BEH C18 and Cortecs C18+ and particle diameter of 1.7 μ m and 2.7 μ m, respectively, and an Xbridge BEH Shield RP18 column of 50 mm x 4.6 mm I.D., 2.5 μ m, all purchased from Waters (Waters, Milford, MA, USA).

2.4. Software

Response modelling and method optimization were performed using JMP 11.0 (S.A.S Institute Inc, Cary, NC, USA) and Matlab (Version: 8.0.0.783 (R2012b), The Mathworks).

3. Results and discussion

To our knowledge, ICH guideline Q8(R2) [6] is the unique guideline dealing with the implementation of QbD principles to the pharmaceutical development. As analytical development is a key part of pharmaceutical development, ICH terminology (e.g. critical quality attributes, critical process parameters) was adapted to analytical methods throughout the manuscript. In literature, ICH terminology has been widely applied to analytical developments based on QbD principles [8,9,29,30].

3.1. Analytical target profile (ATP)

The first step was to define the objectives of the method development, called the ATP. The HPLC method should allow the simultaneous separation of the 16 compounds of interest in a reasonable analysis time. A unique and specific method was targeted to save time during development and ensure molecule identity, respectively. Indeed, this method will be implemented in industry to facilitate the pharmaceutical development of BPM[®] formulations of each of the 16 molecules.

As shown in Fig. 1, the majority of active compounds has acido-basic properties. Some molecules are acidic with pKa varying from 4.0 and 4.6, except for paracetamol which is a very weak acid with acidic pKa of 9.4. Others are basic with pKa ranging from 6.6 to 9.1. Three compounds possess no acido-basic properties: artemether, melatonin, and simvastatin. The range of hydrophobicity is large

with log P values ranging from 0.5 for paracetamol, the most polar compound, to 5.4 for atorvastatin, the most hydrophobic one.

Given the high number of compounds to be separated and the wide diversity in structures and physico-chemical properties (Fig. 1), the development of a specific and robust method in reversed-phase liquid chromatography (RP-HPLC) would require substantial time and labour if a monovariate approach was used by changing one separate factor at a time without considering the fact that the effect of possible interactions between factors would not be detectable.

In HPLC, a high number of factors can impact the quality of separation, mainly the stationary phase (nature of grafted functional group, type of bonding, type of silica and end-capping technology) and the mobile phase: isocratic or gradient elution mode, composition of mobile phases (proportion and type of organic solvent, nature and ionic strength of buffer), initial hold time in gradient, gradient slope, and flow rate. Other factors such as column temperature, sample composition, injection volume, and detection parameters can also affect method performances. Thus, to increase the probability to find experimental conditions that allow fulfilling the requirements stated in the ATP, a high number of parameters should be considered during method optimization.

DOE is recognized as an efficient tool to investigate simultaneously a high number of factors in a limited number of trials. However, if the number of factors to be investigated is too high, even DOE leads to an unreasonable number of experiments. A sequential methodology was therefore followed. Based on prior knowledge, potentially high risk experimental factors were preliminarily identified. Among them, the stationary phase and organic solvents were selected during a first scouting phase, while, remaining high risk factors were simultaneously investigated owing to screening designs to identify CPPs.

3.2. Scouting phase: column and organic solvent selection

In RP-HPLC, it is well known that column chemistry, organic solvent nature, and pH of mobile phase are among the most relevant parameters.

Three columns with different stationary phases were tested: Acquity BEH C18 and Xbridge BEH Shield RP18 columns, both based on hybrid silica but the latter including an embedded polar group increasing the polarity of its surface, and Cortecs C18+ column based on core-shell technology with a positively charged surface. These columns were selected to reduce the interactions between basic compounds and residual silanols. Indeed, BEH technology decreases the number of residual silanols at the surface of the stationary phase while grafting positively charged groups, silanols preferably interact with these groups instead of basic compounds. For this evaluation phase, an exploratory gradient from 5 to 95% of ACN with a 3%/min gradient slope at a temperature of 30 °C was used. Two pH values for the aqueous phase were tested, 2.6 and 6.5, using respectively 20 mM formic acid or ammonium formate.

Although the elution window was similar, very different elution orders were obtained depending on the stationary phase and the pH value, highlighting large differences in the selectivity of columns.

Whatever the pH value, for basic compounds, i.e. domperidone, doxylamine, metopimazine, midazolam, ondansetron, and quinine, higher peak asymmetry values were recorded on Acquity BEH C18 and Cortecs C18+ columns compared to Xbridge BEH Shield RP18.

On Acquity BEH C18 column, peak symmetry for basic compounds was improved with pH: between 1.6 and 4.1 at pH 2.6 and between 1.3 and 2.8 at pH 6.5, probably due to a lower proportion of positively ionized molecules as pH is getting closer to the pKa values of basic molecules, thus reducing unwanted secondary interactions between protonated bases and the deprotonated residual silanols. The contrary is observed on Cortecs C18+ as better peak symmetry

was noticed at pH 2.6 (between 1.4 and 2.8) compared to pH 6.5 (between 1.5 and 3.3). As a matter of fact, lower pH values reduce the proportion of deprotonated silanols, thus decreasing electrostatic interactions between embedded positively charged groups and deprotonated silanols hence leading to a repulsion between embedded cationic groups and protonated bases. Compounds do not interact anymore with the stationary phase, improving the shape of peaks. Electrostatic interactions with residual silanols at the surface of the stationary phase are more important on Cortecs C18+ column compared to Acquity BEH C18 column since BEH technology is not used for the end-capping.

Finally, peak symmetry of basic compounds was much better on Xbridge BEH Shield RP18 with asymmetry values varying from 1.2 and 1.8 whatever the pH values. This may be due to the embedded polar group at the surface of the stationary phase which decreases interactions between analytes and stationary phase. Moreover, this column benefits from the BEH technology, so the number of free silanols at its surface is low, limiting undesirable interactions between analytes and silanols.

As better peak shape was obtained on the Xbridge BEH Shield RP18 column, this column was retained for the next steps of the method development.

The nature of the organic solvent is also an important parameter as it can change the retention of the analytes and modify the selectivity. The two organic solvents investigated were ACN and EtOH. EtOH is one of the best green solvent candidates: its low toxicity and its sourcing from renewable sources reduce risks by minimizing the environmental impact and health hazards. Moreover, EtOH has a cost compatible with a routine use. Drawbacks resulting from the use of EtOH in HPLC can be a poor detectability of compounds without UV chromophore, e.g. artemether, and high backpressures due to its high viscosity [25–27]. Moreover, EtOH can lead to lower peak efficiency than ACN with peak efficiency values decreasing faster with the flow rate using EtOH compared to ACN [5]. However, regarding the various advantages of EtOH, this solvent was tested in this QbD development to compare chromatographic performances achieved using EtOH and ACN.

3.3. Screening designs

Screening designs are used to identify among a large number of factors, the ones that have a significant impact (CPPs) on the responses of interest. Given the large range of hydrophobicity of the 16 compounds, gradient mode was selected to be able to achieve a separation. Using Plackett-Burman designs, the effects of 5 factors were simultaneously investigated: the pH of the aqueous phase, the flow rate of mobile phase, the gradient slope to increase from 5% to 80% of organic solvent, the initial isocratic plateau expressed as mobile phase volume, and the column temperature. As a matter of fact, after the two first variables, i.e. column chemistry and organic solvent, these chromatographic parameters are also known to be likely to affect the quality of the separation of the 16 compounds studied. It may be not so obvious for the flow rate parameter. However, in a previous study, this factor was found to have a negative impact on peak efficiency of statins using ethanol as organic modifier [5]. Consequently, the flow rate factor was included in the screening study of both solvents for comparison purposes.

One screening design was carried out for each organic solvent, ACN and EtOH, corresponding to a total of 24 experiments. The variation domains of the factors were chosen based on the experiments run in the scouting phase: pH, 2.6–6.5; flow rate, 0.5–0.8 mL/min for EtOH and 0.5–1.0 mL/min for ACN; gradient slope, 2–5 %/min; volume of the isocratic step, 500–1000 μ L; temperature, 15–40 °C. Using Plackett-Burman designs, a linear relationship between factors and responses was hypothesized so that main effects of factors could be assessed. Retention times (t_R) of the

Table 1

Estimated model parameters and p -values ($\text{Pr}(>|t|)$) of the significance tests for t_R modelling of the studied compounds in ethanol-based mobile phases. * and bold values in Tables correspond to significant effects with a type one error risk of 1%.

Compounds	pravastatin		atorvastatin		rosuvastatin		fluvastatin		ketoprofen		diclofenac	
	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$
Intercept	14.000	<.0001*	19.640	<.0001*	15.229	<.0001*	20.101	<.0001*	14.383	<.0001*	18.898	<.0001*
Slope	-4.163	<.0001*	-6.465	<.0001*	-4.614	<.0001*	-6.556	<.0001*	-4.108	<.0001*	-5.934	<.0001*
pH	-1.198	0.0018*	-0.873	0.0045*	-1.073	0.0029*	-0.991	0.0041*	-2.452	0.0007*	-2.559	0.0006*
Flow rate	-0.777	0.0134	-0.862	0.0048*	-0.833	0.0094*	-0.905	0.0063*	-0.732	0.1061	-0.754	0.1007
Temperature	-1.121	0.0025*	-1.095	0.0015*	-1.267	0.0012*	-1.191	0.0017*	-1.439	0.0096*	-1.499	0.0084*
Isocratic step volume	0.439	0.0983	0.485	0.0493	0.502	0.0644	0.490	0.0683	0.302	0.4625	0.289	0.4862
Compounds	paracetamol		artemether		melatonin		simvastatin		doxylamine		ondansetron	
	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$
Intercept	3.508	<.0001*	20.886	<.0001*	9.507	<.0001*	22.606	<.0001*	5.192	<.0001*	8.730	<.0001*
Slope	-0.077	0.3435	-6.592	<.0001*	-1.979	<.0001*	-7.596	<.0001*	-0.980	0.0282	-1.863	0.0005*
pH	-0.0139	0.8594	-0.011	0.9545	-0.019	0.9103	-0.037	0.8123	3.399	<.0001*	1.980	0.0004*
Flow rate	-0.755	<.0001*	-1.246	0.0004*	-1.075	0.0006*	-1.079	0.0004*	-1.219	0.0117	-1.296	0.0035*
Temperature	-0.706	<.0001*	-0.964	0.0017*	-1.130	0.0005*	-0.822	0.0015*	0.039	0.9135	-0.620	0.0677
Isocratic step volume	0.188	0.0471	0.671	0.0097*	0.609	0.0103	0.611	0.0066*	0.599	0.1290	0.844	0.0231
Compounds	quinine		metopimazine		midazolam		domperidone					
	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$				
Intercept	7.614	<.0001*	11.724	<.0001*	12.901	<.0001*	13.595	<.0001*				
Slope	-1.561	0.0177	-3.303	<.0001*	-3.572	0.0004*	-4.005	<.0001*				
pH	3.704	0.0003*	1.406	0.0016*	3.611	0.0003*	2.928	0.0005*				
Flow rate	-1.553	0.0180	-1.037	0.0069*	-1.596	0.0182	-1.296	0.0229				
Temperature	-0.087	0.8623	-0.921	0.0117	-0.206	0.6929	-0.74	0.1337				
Isocratic step volume	0.902	0.1101	0.843	0.0170	1.054	0.0777	1.070	0.0461				

16 APIs and 15 time differences (S criterion) were considered as characteristic responses of chromatographic performance. The 15 time differences corresponded to time intervals between successive peaks taking into account the order of elution obtained for the first tested conditions.

Good determination coefficients (R^2) were obtained for t_R (between 0.93 and 0.99) and S criteria (between 0.90 and 0.99). Models were significant according to the analysis of variance test. The model adequacy was further confirmed by the residuals which proved normally distributed.

The significance of the individual model coefficients was evaluated using a t -test (comparing the coefficient estimate to its standard-deviation) with a type one error risk of 1%. The estimated model coefficients and p -values of the significance tests for t_R and time differences modelling are presented in Tables 1 and 2 respectively, for ethanol media and in Tables S1 and S2, respectively, for ACN media.

The graphical analysis of the effects showed that pH and gradient slope had the most significant effects on t_R whatever the organic solvent used (Table 1 and Table S1). Indeed, pH is a fundamental parameter since it impacts the retention of acidic and basic compounds with a respective decreasing or increasing variation with respect to pH. On the contrary, neutral compounds (artemether, melatonin, and simvastatin) or compounds with high acidic pKa value such as paracetamol, showed no t_R variation with pH.

Whatever the organic solvent, gradient slope had a significant negative effect on t_R of compounds except for the first eluting compounds, i.e. those eluting before or at the beginning of the gradient such as paracetamol, doxylamine, and quinine.

Finally, in ACN media, the flow rate was also a critical parameter to optimize retention of molecules as it affected 14 over 16 t_R .

On the contrary, in EtOH media, the flow rate was not found to be significant as only 2 t_R were affected by this factor. This observation may be explained by the variation domain of the flow rate which was larger in ACN than in EtOH. Indeed, using EtOH, the

higher viscosity of mobile phases led to higher pressures, limiting the increase in the flow rate.

In EtOH media, the column temperature turned out to be a critical parameter on retention of the majority of compounds with a negative effect for 10 compounds over 16. To the opposite, in ACN, the temperature was the less critical parameter affecting only 2 t_R .

Finally, the impact of the volume of the isocratic step on t_R was low in ACN (5/16 compounds) and very low in EtOH (2/16 compounds) based-mobile phases.

On separation criterion S, pH was the most critical factor followed by gradient slope in both EtOH and ACN media (Table 2 and Table S2).

To sum up, 3 CPPs were identified for each organic solvent: pH, gradient slope, and flow rate for ACN, and pH, gradient slope, and temperature for EtOH.

3.4. Optimization designs

The previously identified CPPs were further studied by response surface methodology. Central composite designs were carried out to model CQAs as function of CPPs through a second-degree model. They were composed of a two-level full factorial design of 8 points, a star design of 6 points, and a center point replicated 6 times to estimate the experimental error necessary to test for lack of fit [31], leading to a total of 20 experiments for each organic solvent. A random order was followed to minimize the influence of time-related uncontrolled factors on the CQAs. The distance α between the center points and the star design points was fixed at $8^{1/4} = 1.68$ to achieve isovariance criterion by rotation (equal variance of prediction in all directions). Consequently, each factor was examined at 5 levels ($-1.68, -1, 0, +1, +1.68$).

Using ACN as organic solvent, variation domains of factors were: pH, 2.6–8.6; gradient slope, 2–5 %/min, and flow rate, 0.5–1.5 mL/min, while for EtOH, factors were varied over the following ranges: pH, 2.6–8.6; gradient slope, 2–5 %/min, and temperature, 15–40 °C.

Table 2
Estimated model parameters and p -values ($\text{Pr}(>|t|)$) of the significance tests for the 15 time differences (S criterion) modelling in ethanol-based mobile phases. * and bold values in Tables correspond to significant effects with a type one error risk of 1%.

S criterion	doxylamine/paracetamol		paracetamol/quinine		quinine/ondansetron		ondansetron/melatonin		melatonin/midazolam	
Term	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$
Intercept	-2.3168	0.0005*	3.7166	0.0002*	0.5032	0.0677	-0.0459	0.8597	2.9495	0.0009*
Slope	0.9978	0.0264	-1.4073	0.0198	-0.2017	0.4068	0.0866	0.7397	-1.5045	0.0211
pH	-3.6142	<0.0001*	3.7108	0.0002*	-1.9702	0.0001*	-2.1933	0.0001*	3.5655	0.0003*
Flow rate	0.6048	0.1266	-0.7543	0.1422	0.3620	0.1605	0.2739	0.3131	-0.4688	0.3712
Temperature	-0.8233	0.0523	0.6079	0.2223	-0.4820	0.0770	-0.4446	0.1242	0.9583	0.0957
Isocratic step volume	-0.4650	0.2217	0.6893	0.1737	-0.1100	0.6439	-0.3114	0.2573	0.4508	0.3887
S criterion	midazolam/metopimazine		metopimazine/domperidone		domperidone/pravastatin		pravastatin/rosuvastatin		rosuvastatin/ketoprofen	
Term	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$
Intercept	-1.5833	0.0013*	1.3257	0.0002*	-0.0658	0.9134	0.8103	<0.0001*	-1.2738	0.0008*
Slope	0.3669	0.2362	-0.6032	0.0100	-0.0666	0.9123	-0.3788	0.0001*	0.6058	0.0253
pH	-2.2069	0.0002*	1.4407	0.0001*	-4.2171	0.0003*	0.0891	0.0819	-1.3968	0.0005*
Flow rate	0.5884	0.0793	-0.2608	0.1603	0.5736	0.3604	-0.0214	0.6336	0.1204	0.5778
Temperature	-0.7126	0.0431	0.1423	0.4156	-0.3208	0.5999	-0.1289	0.0234	-0.1489	0.4943
Isocratic step volume	-0.2114	0.4770	0.1650	0.3500	-0.6266	0.3211	0.0504	0.2821	-0.2094	0.3458
S criterion	ketoprofen/atorvastatin		atorvastatin/artemether		artemether/fluvastatin		fluvastatin/diclofenac		diclofenac/simvastatin	
Term	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$	Estim.	$\text{Pr}(> t)$
Intercept	4.6940	<0.0001*	0.7685	<0.0001*	-1.2483	<0.0001*	-1.6966	0.0002*	3.1796	<0.0001*
Slope	-2.2187	<0.0001*	-0.0013	0.9872	0.1648	0.2390	0.7399	0.0149	-1.5476	0.0030*
pH	1.4727	0.0008*	0.7953	<0.0001*	-1.0366	0.0002*	-1.6224	0.0003*	2.4173	0.0003*
Flow rate	-0.0533	0.8295	-0.3440	0.0050*	0.3884	0.0216	0.2003	0.3961	-0.2753	0.4260
Temperature	0.3565	0.1833	0.1582	0.0942	-0.2251	0.1244	-0.2849	0.2413	0.7218	0.0665
Isocratic step volume	0.1673	0.5067	0.1568	0.0965	-0.1876	0.1872	-0.2338	0.3272	0.3456	0.3250

The ranges of variation of gradient slope and temperature were kept the same as for the previous screening step. However, wider scales of pH and flow rate were investigated to expand the studied experimental domain and increase the probability to find conditions allowing a complete separation between the 16 compounds. Others factors which were not identified as CPPs were kept constant during the optimization study. In both ACN and EtOH media, an isocratic step was performed before the gradient for industrial transfer reasons and fixed at 500 μL to reduce analysis time. Temperature was fixed at 25 $^{\circ}\text{C}$ for ACN study, while flow rate was set at 0.8 mL/min for the study in EtOH to increase the speed of analysis.

As stated above, the peak-start (t_i^{start}), peak-end (t_i^{end}), and also peak retention (t_R) times of the 16 compounds were modelled as second-degree functions of the CPPs. These models could be considered unbiased according to the test for lack of fit with a type one error risk of 5%, except for 3 compounds (doxylamine, ondansetron, and quinine).

Using these models, S criteria, i.e. time differences between peaks were computed. More precisely, the experimental domain was divided in elementary cubes ($17 \times 17 \times 17 = 4913$ cubes) and, for each of them, the time intervals between all possible pairs of peaks were estimated using the models of peak-start and peak-end times:

$$\Delta t_{ij}(k) = \max(t_i^{\text{start}}(k) - t_j^{\text{end}}(k), t_j^{\text{start}}(k) - t_i^{\text{end}}(k))$$

where i and j index the peaks ($i, j = 1$ to 16, $i < j$, i.e. 120 different peak pairs) and k indexes the elementary cubes ($k = 1$ to 4913). In order to prevent prediction errors due to the inaccurate modelling of the three compounds mentioned previously, an additional constraint was applied: in zones where the order $t_i^{\text{start}} < t_R < t_i^{\text{end}}$ was not obtained for all compounds ($i = 1$ to 16), the models were considered unreliable and these zones were excluded from the analysis.

A desirability analysis was finally applied on the smallest time interval in the regions meeting the peak non-overlapping constraint (i.e. the regions where a complete separation between the 16 peaks was predicted) and reliability conditions. Optimal con-

ditions, for which the time interval of the critical peak pair was the highest, were then predicted by maximizing the desirability value. In ACN media, no experimental conditions complying with the non-overlapping constraint between peaks were found, and consequently no optimum point could be predicted.

On the contrary, using EtOH as organic modifier in mobile phase, some conditions could be determined for which a complete separation between all peaks was predicted. Optimal conditions were determined by maximizing the desirability value and corresponded to a gradient slope of 2.57 %/min, a pH of 4.85, and a temperature of 33.7 $^{\circ}\text{C}$. Representations of the 3D experimental domain with DOE points, calculated desirability values, and predicted optimal point are shown in Fig. 2. Given the desirability values (Fig. 2B), several zones where the separation was predicted were evidenced, probably due the large experimental domain studied. The experimental domain was defined as the zone containing the optimum point and the robustness inside was then experimentally tested. Note that due to large number of constraints (16 compounds studied), the size of the zone where both model consistency and absence of peak overlap were obtained was very reduced, so that we were not able to define a minimum quality, as advised by Lebrun et al. [24] for example.

3.5. Robustness experimental validation

The chromatogram recorded under the predicted optimal conditions is provided in Fig. 3.

At the optimum, requirements stated in the ATP were fulfilled since the 16 peaks were baseline resolved with resolutions superior to 2.4.

Then, to evaluate the robustness of the method and find the experimental domain where all peaks are separated, CPPs were deliberately varied owing to a new Plackett-Burman design. Variation ranges tested corresponded to 0.2 coded units around the optimum point: from 4.82 to 5.02 for pH, from 2.47 to 2.66 %/min for gradient slope, and from 32.9 and 34.5 $^{\circ}\text{C}$ for temperature. No

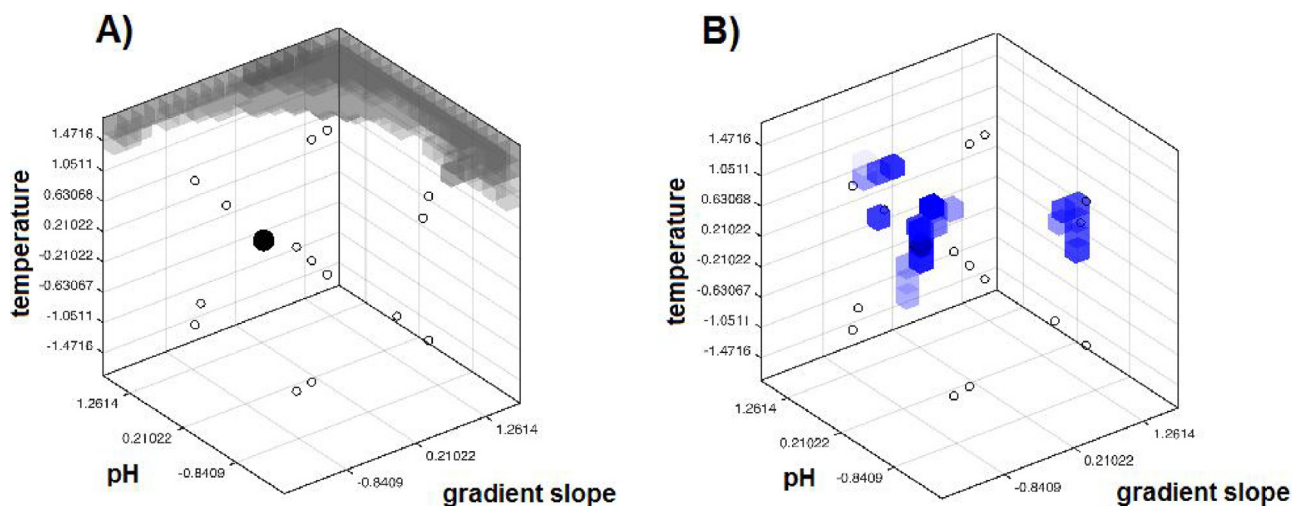


Fig. 2. Representations of the 3D experimental domains. A) Experimental DOE points (small circles) and a dark zone indicating where the models are considered unreliable. B) Desirability values for the conditions satisfying the non-overlapping constraints: the darker the color, the higher the desirability. The big black point corresponds to the highest value of desirability and hence to the predicted optimum point (gradient slope = -1.04 , pH = -0.42 , temperature = 0.83).

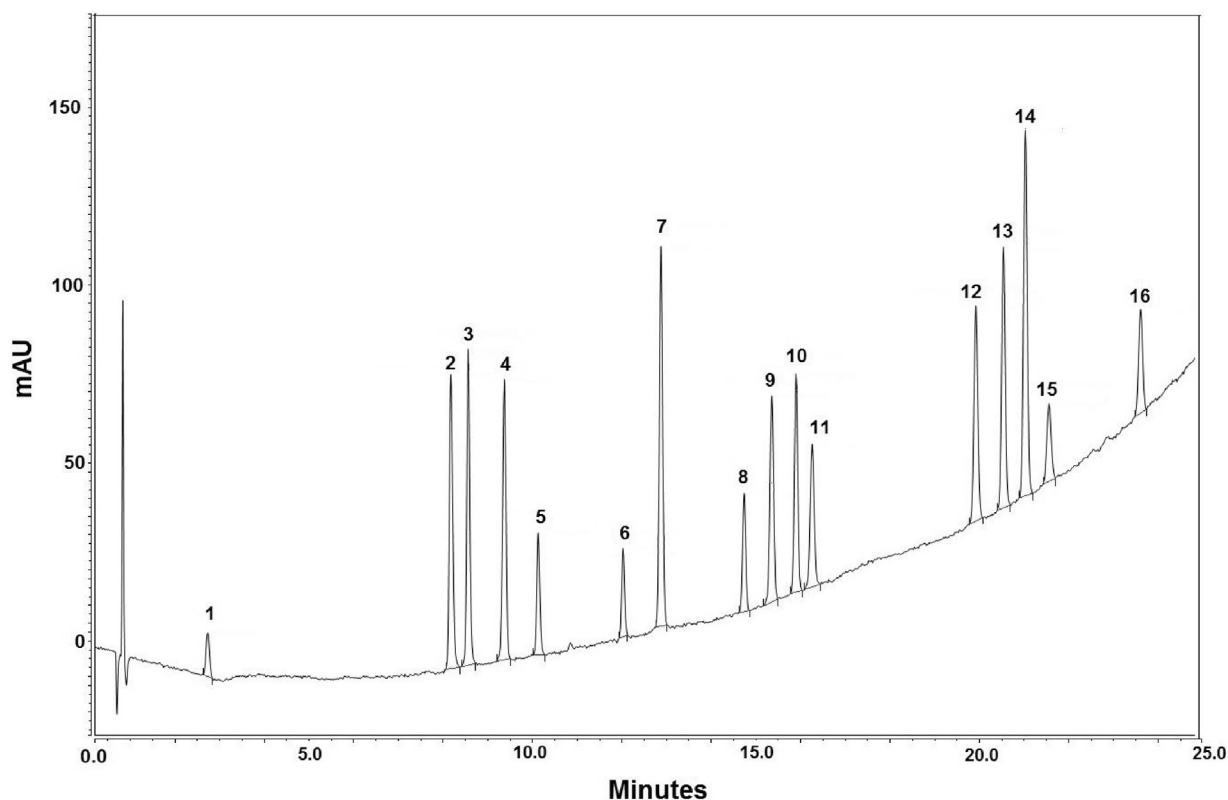


Fig. 3. Chromatogram recorded on the Xbridge BEH Shield RP18 column 50 mm \times 4.6 mm I.D., 2.5 μ m at 33.7 $^{\circ}$ C with an initial hold of 0.6 min (corresponding to a 500 μ L isocratic step volume) at 5% EtOH and a gradient from 5% to 80% of EtOH with a slope of 2.57 %/min. Mobiles phases composed of acetate buffer 20 mM at pH 4.85 for the aqueous phase. Flow rate: 0.8 mL/min. Detection at 210 nm. Peak identification: 1 = paracetamol, 2 = doxylamine, 3 = ondansetron, 4 = melatonin, 5 = quinine, 6 = metopimazine, 7 = domperidone, 8 = pravastatin, 9 = ketoprofen, 10 = rosuvastatin, 11 = midazolam, 12 = diclofenac, 13 = atorvastatin, 14 = fluvastatin, 15 = artemether, 16 = simvastatin.

change in elution order was observed inside this zone and, for all tested conditions, the 16 peaks were always baseline resolved with resolutions superior or equal to 2.2. Consequently, inside this experimental subspace, the separation method was robust.

4. Conclusions

In this work, the relevance of QbD was demonstrated through the development of a specific and robust UHPLC method for the

simultaneous analysis of 16 APIs of very different physico-chemical properties. Each key step of QbD methodology was investigated and an original optimization approach based on the systematic evaluation of time intervals between all possible pairs of peaks over the whole experimental domain, was followed. Under optimized conditions, the 16 APIs were baseline resolved using ethanol in mobile phases, one of the best green solvent candidate. Due to the low toxicity of ethanol and its sourcing from renewable sources, such method reduces environment impact and analyst exposure. The

experimental domain of the method was established and robustness to parameters changes was proved inside. In a near future, this analytical method will be quantitatively validated for each API individually in pharmaceutical forms prior to solubility tests and industrial transfer. The results presented in this paper highlight the fact that QbD approach is an essential tool for the optimization and robustness assessment of new methods for the chromatographic analysis of complex mixtures in pharmaceutical sciences.

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