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Angéline Noireau, Elise Lemasson, Fabien Mauge, Anne-Marie Petit, Sophie Bertin, et al.. Purification of drug degradation products supported by analytical and preparative supercritical fluid chromatography. *Journal of Pharmaceutical and Biomedical Analysis*, 2019, 170, pp.40 - 47. 10.1016/j.jpba.2019.03.033 . hal-03484432

HAL Id: hal-03484432

<https://hal.science/hal-03484432>

Submitted on 20 Dec 2021

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Purification of drug degradation products supported by analytical and preparative supercritical fluid chromatography

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ABSTRACT

A stressed degradation (oxidation) was employed to produce metabolites from an active pharmaceutical ingredient (API) with large molecular weight (about 900 g/mol). An analytical chromatographic method was desired to compare the products generated by different degradation methods while a multi-gram-scale preparative chromatographic method was necessary to purify the produced metabolites. Supercritical fluid chromatography (SFC) was selected for both tasks as no other chromatographic method had achieved the resolution of the API and metabolites (two isomeric mono-oxide species and one di-oxide). First, an analytical-scale method was developed with ultra-high performance supercritical fluid chromatography (UHPSFC). Achiral stationary phases containing sub-2 µm fully porous particles or sub-3 µm superficially porous particles, and chiral phases containing 3 and 5 µm fully porous particles were selected for a first screening with gradient elution (carbon dioxide – methanol containing additives). The stationary phase providing the most promising results was ACQUITY Torus 2-PIC (100 x 3 mm, 1.7 µm, Waters). A central composite design (CCD) was conducted to optimize the gradient program and oven temperature. Final gradient conditions were as follows: 50 to 70 % methanol in 3.8 min with oven temperature set at 36°C, back-pressure set at 11 MPa and flow-rate at 0.8 mL/min. The optimized method was employed to analyze samples obtained with different degradation conditions. Then the method was adapted and transferred to preparative-scale SFC on a 5 µm-particles Torus 2-PIC stationary phase (150 x 30 mm). The method was modified to comprise an isocratic step followed by a gradient, favoring peak shape of the last eluting compound and minimal volume of collected fractions. Batch injections in gradient mode were carried out to purify six grams of crude product.

Keywords: Degradation products; Supercritical fluid chromatography; Preparative SFC; Enhanced Fluidity Liquid Chromatography

Abbreviations:

ADMET	Absorption, distribution, metabolism, excretion, toxicity
API	Active pharmaceutical ingredient
CCD	Central composite design
DoE	Design of experiments
EFLC	Enhanced fluidity liquid chromatography
EtOH	Ethanol
HPLC	High-performance liquid chromatography
iPrOH	Isopropanol
MeOH	Methanol
SFC	Supercritical fluid chromatography
UHPSFC	Ultra-high performance supercritical fluid chromatography

1. Introduction

In the course of drug development, impurities, degradation products and possible metabolites [1] must be determined and purified in order to assess their toxicity for the human body. Degradation under stressed conditions are part of stability testing [2], governed by FDA and ICH guidelines [3]. The aim is to understand how a drug product will vary with time, using extreme conditions to mimic different climatic areas [4]. This degradation study may also have the purpose of producing metabolites observed in in-vivo degradations (or metabolism) when absorbing the drug, as part of the ADMET parameters (absorption, distribution, metabolism, excretion and toxicity) [5]. To identify degradation products or metabolites, high-performance liquid chromatography (HPLC) is most widely used [6]. When the degradation products and metabolites are identified, it is also necessary to obtain them in sufficient quantities for activity testing. For this purpose [7], but also to produce pure fractions for identification [8] or to generate individual enantiomers [9], preparative chromatographic techniques are most useful.

Supercritical fluid chromatography (SFC) is close to HPLC except that the main component of mobile phase is not a liquid solvent but a compressible fluid. The mobile phase composition most frequently employed in SFC is composed of a mixture of pressurized carbon dioxide [10] and a short-chain alcohol like methanol [11,12]. CO₂ and the organic co-solvent may be mixed in all proportions, although most SFC applications report mobile phase compositions with a larger proportion of CO₂ than co-solvent. When the proportion of solvent is larger than the proportion of carbon dioxide, the technique is often called “enhanced-fluidity liquid chromatography” (EFLC) [13]. This type of mobile phase composition was mostly employed by the group of S. Olesik, typically to analyze polar, water-soluble compounds such as amino acids [14], nucleosides [15] or protein [16]. When the compounds

have moderate polarity, the simple use of polar co-solvents and additives is often sufficient to achieve satisfying elution, as was already discussed by T.A. Berger long ago [17].

The principal advantages of SFC mobile phases compared to HPLC ones, are the low fluid viscosity allowing for high efficiencies at high flow rates, and the green features of CO₂. D. Speybrouck and E. Lipka even said that “greenness is not the icing on the cake but the cake itself” [18]. CO₂ is recyclable, produced as a side-product from other industries, therefore has a low cost, is non-flammable and less toxic than most other solvents employed in HPLC [10]. Most of these useful characteristics are desirable, not only at the analytical scale but also at preparative scale, particularly for enantioseparations [9,19]. When employing small-particle fully porous (sub-2 µm) or superficially porous (sub-3 µm) stationary phases, combined with modern instruments offering reduced dead volumes, SFC offers comparable features to ultra-high performance liquid chromatography (UHPLC) and may be termed “ultra-high performance supercritical fluid chromatography” (UHPSFC). The separations obtained with UHPSFC have high efficiency and can be obtained very rapidly, especially as the low fluid viscosity is not causing so high pressures as in UHPLC.

In this project, the product of interest is an active pharmaceutical ingredient (API), potential future drug (stage I of development) with rather large molecular weight (about 900 g/mol). This product was subjected to stressful degradation, and oxidation in particular. Partial structures of the API and oxidation products can be seen in Fig. 1, to observe the position of oxidation sites relative to the whole molecule. Therefore, the objective of this project was to analyze the products of stressful degradation and to obtain enough purified oxidized compounds for future activity testing. Preliminary experiments had shown that liquid chromatography (in the reversed-phase or normal-phase modes) was unsuccessful to achieve the resolution of the API from its oxidation products. Indeed, because the molecule is rather large, it was difficult to solubilize and the structural difference caused by the introduction of one or two oxygen atoms in the large molecular structure was very small and difficult to resolve. Because SFC is known to be effective in the separation of closely-related species [20,21], it was investigated to achieve this task.

First, an analytical method was developed to analyze samples from different degradation experiments conducted on the same active ingredient. The analytical conditions were optimized using a Design of Experiments (DoE). This chemometric approach is very useful to determine the best experimental parameters for an analysis. It allows evaluating combined interactions between parameters within a short space of time (couple hours) [22]. The optimal analytical method then needed to be adapted and scaled up to purify a few grams of the oxidized products. Robustness around the optimal conditions (+/- 1%) was also examined to ensure a successful transfer [23].

2. Material and methods

2.1. Chemicals and solvents

The API and degradation products were produced at Servier Research laboratory in Orleans, France. Their structure is confidential but their molecular weight is around 900 g/mol. Oxidative degradation with hydrogen peroxide was carried out according to ICH guidance [3].

At the University of Orleans, for the analytical-scale method development:

Solvents employed were HPLC-grade methanol (MeOH), ethanol (EtOH) and isopropyl-alcohol (iPrOH) provided by VWR (Fontenay-sous-Bois, France). Air Liquide (Paris, France) provided carbon dioxide of industrial grade $\geq 99.7\%$. Ammonium acetate powder $\geq 98\%$ was supplied by Merck, formic acid $\geq 99\%$ was obtained from VWR (Fontenay-sous-Bois, France) and an Elga Purelab Flex station from Veolia (Antony, France) provided ultra-pure water.

At Servier Research laboratories, for the preparative-scale method and analysis of fractions:

Solvents used were MeOH and iPrOH for analysis-ACS-Reagent, provided by CARLO ERBA. Honeywell supplied methylene chloride $> 99.9\%$ (used for product dissolution). Ammonium acetate ($>98\%$) was obtained from ACROS and formic acid ($> 98\%$) from Merck. Messer provided carbon dioxide ($\geq 99.995\%$) and ultra-pure water was obtained with a MiliQ® - Advantage A10 system.

2.2. Instrumentation

2.2.1. UHPSFC system

The analytical system used for analytical method development and analysis of fractions was an ACQUITY Ultra Performance Convergence Chromatography™ (UPC²®) from Waters Corporation (Milford, MA, USA). It was equipped with a binary solvent delivery pump, compatible with mobile phase flow rates up to 4 mL/min and pressures up to 414 bar. The system had an autosampler that included partial loop volume injection system, a backpressure regulator and 2-position column oven compatible with 150 mm length columns. Two detectors were available: a photodiode-array (PDA) detector and an ACQUITY QDa® single-quadrupole mass spectrometric (MS) detector with electrospray ionization source (ESI). An isocratic solvent manager was used as a make-up pump and was positioned before the mass detector. The main flow stream was then split by the on-board-flow-splitter assembly. With this system, a portion of the column flow goes to the backpressure regulator and another (unknown) portion goes to the mass detector. Empower®3 software was used for system control and data acquisition.

2.2.2. Preparative-scale SFC system

An SFC-PICLab PREP 200 from PIC Solution™ (Avignon, France) was employed for the purification of oxidation products. It was a 200 ml/min preparative SFC system designed for 20 and 30 mm i.d. columns. It could stand up to 350 bar and host five columns (or four columns plus bypass). Thanks to a recycling system, and depending on co-solvent percentage, CO₂ should be retrained in supercritical conditions (for instance, more than 95% of CO₂ is recycled with 10% of co-solvent). The system had

five collection tracks, of which one served for waste. SFC PICLAB PREP ONLINE was the software used for system control and data acquisition.

2.3. Stationary phases

In order to determine a stationary phase to achieve the best resolution of API and degradation products, fifteen commercial columns were selected: six chiral and nine achiral stationary phases. Their characteristics are gathered in Table 1. Waters (Guyancourt, France), Phenomenex (Le Pecq, France) Supelco, Thermo Fisher Scientific (Villebon, France), Macherey-Nagel (Hoerd, France) and Chiral Technologies (Illkirch-Graffenstaden, France) kindly provided the columns.

2.4. Chromatographic conditions

The screening of the different columns was performed with a large elution gradient from 10 to 100% of co-solvent in CO₂ in 15 min. Indeed, because the API and oxidation products were strongly retained, it was necessary to elevate the proportion of co-solvent to a high percentage to elute them from the columns. The co-solvent was MeOH containing 2% pure water and 20 mM ammonium acetate (AA). This mobile phase composition was previously defined as being most favorable to the elution of drug candidates with good peak shapes, along with good ESI-MS response [24]. The flow rate was set at 0.5 mL/min with sub-2 μ m columns and 1 mL/min with other column dimensions, to avoid reaching the upper pressure limit before the end of the gradient program. Temperature was set at 25°C and the outlet pressure was fixed at 15 MPa.

Samples were prepared at 1 mg/mL in methanol and 2 μ L were injected. The wavelength of UV detection was set at 210 nm, with frequency set at 20 pts/s and resolution at 1.2 nm.

The mass detector unit was pre-optimized by the manufacturer. Ion source temperature was fixed at 120°C, probe temperature at 600°C, scan rate at 8 pts/s, capillary voltage at 0.8 kV, cone voltage at 15 V and sampling frequency at 8 Hz. Nebulizing gas was nitrogen. The make-up solvent composition was 98% MeOH, 2% pure water plus 1% formic acid with a 0.45 mL/min flow. The analytes were detected in positive and negative electrospray ionization mode (m/z 400-1000).

2.5. Mobile phase optimization

After selecting the best stationary phase from the screening experiments (ACQUITY Torus 2-PIC), a central composite design (CCD) was conducted to optimize the gradient program. Flow-rate was set at 0.8 mL/min and backpressure at 11 MPa in order to not generate system overpressure. Because three analytical parameters had to vary (final co-solvent percentage, gradient time and oven temperature), the CCD contains a factorial design with $2^3 = 8$ points (angles of the cube), six star points and a central point with six replicates to assess the experimental error. The distance between the center of the design space and a factorial point was ± 1 unit whereas the distance between the center of the design space and a star point is $\alpha = 2^{3/4} = 1.682$ with α is the isovariance criterion by rotation [25]. Therefore,

each of the three analytical parameters was explored at five levels: -1.682; -1; 0; +1; +1.682 (see Table 2). The software used to assist in this work was JMP® trial version, (S.A.S Institute Inc, Cary, NC, USA).

Judging from the screening experiments, the initial co-solvent percentage was fixed at 50%. Again this unusual initial percentage is due to the high molecular weight (~900 g/mol) of the API and degradation product. Overall, 20 experiments were conducted, in a random order generated by the software.

Optimum calculation:

For each experiment, the separation criterion (S) was used (instead of resolution) to assess the separation quality of two successive peaks [26]. It is defined as the difference between the retention time at the beginning of the $n+1$ peak ($t_{B\ n+1}$) and the retention time at the end of the n peak ($t_{E\ n}$). Peaks are baseline resolved if S is positive.

$$S = t_{B\ n+1} - t_{E\ n} \quad (1)$$

When the optimal point was found, because the result was still not considered sufficiently good, the separation was further improved with the modification of co-solvent nature (MeOH and iPrOH). The final mobile phase co-solvent was MeOH-iPrOH 70:30 (v/v), comprising 20 mM ammonium acetate and 2% water.

Then, the robustness in optimized conditions was explored with variations of each of the three optimized parameters (final solvent percentage, gradient time, temperature) of more or less 1%. Thus six additional experiments were done to assess the changes caused by small variations around the optimal point. The experiments and results are presented in Tables S1 and S2, in supplementary material.

2.6. Purification conditions in preparative SFC

The SFC-PICLab PREP 200 was used to purify the active pharmaceutical ingredient (API) from its oxidized degradation products. ACQUITY UPC² Torus 2-PIC column (150 x 30 mm, 5 μ m) was employed and the mobile phase composition was the one optimized at the analytical scale. In preparative SFC, stacked injection in isocratic elution mode is preferred to increase productivity [9]. In addition, the lowest proportion of solvent is desirable to limit solvent consumption and evaporation time. Therefore, methods in isocratic elution mode were also tested with 40%, 45%, 50%, 55% and 60% of co-solvent. It turned out that a rather good separation with poor peak shapes were obtained with 45% of co-solvent in 15 minutes. However, in the present case, the last peak (di-oxidized compound) showed a significantly tailing peak, probably due to the low solubility of this compound. In order to improve the peak shape and minimize the volume of collected fractions for this peak, a

second gradient program was designed, which prevented the use of stacked injections. First, the program included an isocratic step with 45% co-solvent during 10 min. Then, the co-solvent proportion was raised to 65% in 1 min and maintained during 5 min to ensure the elution of the last compound. Thanks to high diffusivities in the mobile phase, due to the presence of a large portion of pressurized carbon dioxide, the column was reconditioned at 45% for only 4 min before the next injection. Cycle time was then 18.1 min. The flow rate was set at 100 mL/min, oven temperature at 40 °C, outlet pressure at 10 MPa and UV detection at 210 nm. Six grams of crude product were dissolved in 15 mL methylene chloride. The injection volume was 300 µL; the injection lasted 10 s.

Fractions quality control:

The solvent from the collected fractions was evaporated with a rotary evaporator and the dry residue was weighted. For purity analysis, ACQUITY UPC² system was used with Acquity Torus 2-PIC (150x4.6 mm, 5 µm) column. The mobile phase was the same as used for compounds purification. The elution gradient profile was: 55% co-solvent from 0 to 5 min, then an increase to 70% from 5 to 10 min and finally re-equilibration at 55% in 0.1 min for a 12 min total run. Flow rate was 2.5 mL/min, oven temperature was fixed at 35 °C and a volume of 3 µL was injected. Detection wavelength was set at 290 nm (instead of 210 nm) because no baseline drift appeared at this wavelength when increasing co-solvent percentage from 55% to 70%. Mass detection was carried out by the QDa[®] with the same previous settings except for the mass range (from 150-700 m/z). Make-up solvent for mass analysis was MeOH/water (90/10) v/v with 0.1% formic acid. Flow rate was 0.5 mL/min.

3. Results and discussion

3.1. Stationary phase selection

Fifteen columns were selected for the initial screening (Table 1). Stationary phases with small particle size (fully porous sub-2 µm or superficially porous 3-µm) were favored, apart from some chiral phases that were not available with such dimensions. Although no chirality is involved in the desired separation, both achiral and chiral stationary phases were selected, as chiral phases are also interesting to separate analytes with small structural differences [27]. The columns selected cover a wide range of selectivities, primarily for polar-type interactions as the separation process should target the number and position of oxygen atoms from the oxidation process. Indeed, it was expected that positional isomers of oxidation products would be produced by the forced degradation. The diversity of selectivities in the chosen columns can be observed from the spider diagram [28] in Figure 2.

The aim of this first screening step was to find one stationary phase that would provide the best separation of the API and all degradation products while maintaining a percentage of co-solvent as low as possible. Indeed, while a large proportion of co-solvent is not causing any issue at the analytical scale, it is not desirable for preparative-scale to have a large proportion of co-solvent because the solvent consumption and waste would rise, and the volume of solvent to evaporate from collected fractions would be unreasonable and time-consuming. At this stage, both UV and mass

spectrometric (MS) detection were employed: UV served at observing resolution between all species in the samples while MS served at identifying the target analytes (API and oxidation products). Indeed, no standard was available to compare to the samples, so the MS spectra were examined. In addition, the UV chromatograms shows additional peaks that need not be purified.

To compare the fifteen different stationary phases, a gradient elution running from 10 to 100% co-solvent was employed. This wide gradient thus comprises the usual domains of SFC and EFLC. The API elution composition (C_e) was calculated according to Eq. 2 and corresponds to the co-solvent percentage required to elute the compound of interest [29] .

$$C_e = C_i + \frac{(C_f - C_i)}{t_G} * (t_R - t_D) \quad (2)$$

C_i and C_f are respectively the initial and final compositions of the gradient, t_G is the gradient time, t_R is the retention time of the compound and t_D is the system dwell time (0.46 min in our case).

As a result of the columns screening (chromatograms in electronic supplementary information, Figure S1), with gradient conditions from 10 to 100% co-solvent, all columns yielding excessive retention (elution composition of API above 50% co-solvent) were excluded: Acquity UPC² HSS C18 SB, Kinetex HILIC, Accucore HILIC, Accucore Amide HILIC, Accucore Urea HILIC, Ascentis Express OH5, Chiralpak IA, IB, IC, ID, IF and IG. The oxidation products were all more retained than the API, and sometimes some of them did not elute at all.

The next step was to look at the number of detected peaks and the separation quality. At this stage, Ascentis Express F5 was excluded because the peaks were broad. ACQUITY UPC² Torus 2-PIC from Waters and Nucleoshell HILIC from Macherey-Nagel were the two stationary phases promising the best results in terms of separation optimization (this is visible in the UV chromatograms in electronic supplementary information, Figure S1). However, the gradient profile was not satisfactory with the Nucleoshell HILIC column because the ammonium acetate breakthrough curve caused a bump in the baseline that happened at the same time as one target peak. Indeed, the additives (ammonium acetate, water) are present at a low but increasing concentration in the mobile phase. At the beginning of the gradient, the additives adsorb on the stationary phase as any other mobile phase component. When the stationary phase is saturated with additives, a small breakthrough curve is visible in the baseline (gradient slope). On stationary phases where ionic interactions are strong (as is the case on the sulfobetaine ligand in Nucleoshell HILIC column), the sudden rise of ammonium acetate concentration in the mobile phase is usually causing the grouped elution of ionic analytes, causing a loss of resolution. Also, because of the highest concentration of ammonium acetate at this moment, it is undesirable to collect a fraction at that time, thus the ACQUITY UPC² Torus 2-PIC was retained as the best column. Moreover, it was important that the selected column was available at preparative scale.

3.2. Design of experiments

Twenty experiments were conducted according to the design of experiments (DoE) described in experimental section to generate optimal gradient and temperature conditions. The whole set of DoE experiments lasted around four hours, taking into account the column equilibration time. The best resolution was desired, together with narrow and symmetric peaks. At the preparative scale, thin and symmetric peaks essentially mean that the time window for fraction collection will be the lowest possible, thereby ensuring more concentrate fractions and less solvent to evaporate to obtain the dry product. Good resolution will also mean that the purity of the fractions collected shall be high. The chromatogram in Fig. 3 represents the separation between the API and degradation products in optimal analytical conditions determined by the DoE. The analytes were identified with the assistance of ESI-MS detection. The first peak, with a 2 minutes retention time, was not interesting for this project so it will not be further discussed. The second peak corresponded to the active pharmaceutical ingredient with a molecular weight (M) near $M \sim 900$ g/mol. The third and fourth peaks were isomeric mono-oxidized degradation products with $M+16$ molecular weight. The last peak had a $M+32$ molecular weight, corresponding to a di-oxidized degradation product.

To generate optimal conditions, five responses were selected and taken into account in the software. Four separation criterions (S), calculated according to Eq. (1), in order to assess the separation quality between the five main peaks, and the asymmetry of the last eluted peak, because it tended to tail significantly.

For each of the five responses, desirability was defined. The highest S value was desired for the four S criterions. For asymmetry criterion, we wanted values the closest to one. For example, iso-response graphs are presented in Fig. 4 for the critical pair of peaks (3 and 4) corresponding to the two mono-oxidized degradation products. These graphs allowed to model the DoE response: temperature vs. final proportion of co-solvent (on the left) and temperature vs. gradient time (on the right). The darkest area corresponds to a response zone where desirability is maximum. The crossing of the dashed black lines represents optimal values (normalized values) determined by DoE. On this figure, it appears that the overall optimum (defined by crossed interrupted lines) is not necessarily the same as the local optimum for each pair of consecutive peaks. The overall optimum is therefore a compromise to satisfy all five responses.

Optimal elution gradient conditions were found as follows: from 50 to 70.3% co-solvent (corresponding to the normalized value 0.052) in 3.8 minutes (normalized value -1) with oven temperature set at 36°C (normalized value +1).

Modification of co-solvent nature:

After the elution gradient optimization with MeOH containing 20 mM AA and 2% water as co-solvent, the two mono-oxide peaks were not sufficiently resolved to hope for a productive purification (Fig. 3). Therefore, it was decided to modify the mobile phase composition, introducing a portion of isopropyl-

alcohol (iPrOH). This solvent is less polar than MeOH and caused increased retention and separation factors (Fig. 5). A MeOH / iPrOH 70/30 (v/v) mixture was finally selected as a middle ground between separation quality, peak shape and run time. The final conditions for this analytical method may not be SFC, but they may be EFLC.

The robustness of the method was assessed as described in experimental section and supplementary material (Tables S1 and S2). It was observed that all parameters observed (retention time, peak area, asymmetry and resolution) remained stable within 2%.

The method then served to compare different samples obtained after different conditions of stressed degradation applied to the same API, prior to scale-up.

3.3. Method transfer to preparative-scale

The optimal SFC (or EFLC) method determined at the previous step was transferred to the preparative scale as described in the experimental section. Four fractions were collected: the API from 5.47 to 6.67 min, two mono-oxidized degradation products from 6.83 to 8.3 min and from 9.2 to 10.7 min and one di-oxidized degradation product from 11.92 to 15.43 min (Fig. 6). The whole purification experiments lasted nearly 20 hours. Overall, 60 L of solvent and 57 L of carbon dioxide were eluted through the system. Actually, the CO₂ consumption was much less than 57 L thanks to the recycling system implemented. According to PIC Solution™, more than 90 % of CO₂ is recycled with 10% of co-solvent and 85% is recycled with 30% of co-solvent. We may thus expect that a lower proportion of CO₂ would be recycled in the present conditions. The collected volumes and masses of dry product obtained for each fraction are indicated in Table 3. Obviously, the volume of solvent collected for the fourth peak (di-oxidized product) is much larger than the others, due to (i) large proportion of this compound in the sample injected and (ii) large proportion of co-solvent in this section of the chromatogram, to elute this peak with reasonably short time frame. It is important to note that such large proportions of solvent are quite unusual for SFC, but were necessary here to separate and elute these high-molecular weight products. In terms of productivity, 306 mg of product were purified in an hour for an average co-solvent consumption of 3 liters. For example, the purified mass is 14 times that of the product purified in a paper from E. Landagaray et al. [22], but it is also 14 times more solvent consuming. In terms of productivity, we have purified about 0.1 kg product per kg of stationary phase per day (kkd), which may be compared to about 0.2 kkd in ref. [22]. While 0.1 kkd is by no means an exceptional productivity, it may however be considered reasonably good [18], especially judging from the difficulty in the separation and the fact that three compounds needed to be collected among other undesirable species. If larger quantities had been desired, further optimization could have been done (flow rate, injected mass and volume, dilution solvent, etc.).

3.4. Fractions analysis

Each fraction was analyzed in order to verify the quality of purified compounds. The results are summarized in Table 3. We noticed that the API collection (fraction 1) was pure at 98.5% whereas for the mono-oxidized degradation products (fractions 2 and 3), the presence of di-oxidized compound (eluted later) was detected up to 2.6%. It was not a problem because the desired purity level of mono-oxidized compounds was achieved (95.8% and 97.5%). The presence of di-oxidized compound in these two fractions may result from further oxidation occurring after the preparative chromatography, perhaps at the evaporation stage but this hypothesis was not verified.

4. Conclusions

Supercritical fluid chromatography at analytical-scale was used to solve the issue in degradation products separation, emerged throughout first trials in HPLC. Thanks to this technique, three degradation products (two isomeric mono-oxidized and one di-oxidized) were isolated from the API. The fast and broad screening of stationary phases and design of experiments quickly provided an optimal analytical method. Because of the large molecular weight of the analytes, inducing limited solubility and strong interactions with the stationary phase, unusual SFC conditions were necessary to obtain this separation, with co-solvent percentage in carbon dioxide up to 70%, which is probably more relevant of enhanced-fluidity liquid chromatography (EFLC). Contrary to some previous reports from Miller and Potter [30], scale-up from ultra-high performance chromatographic conditions to preparative SFC was quite straightforward in this case and allowed efficient purification of all degradation products for further activity testing.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

Waters Corporation is warmly thanked for the support received at the University of Orleans through the Centers of Innovation program. We also thank Régis Guyon (Macherey-Nagel), Dave Bell (formerly at Sigma-Aldrich), Magali Dupin and Marc Jacob (Phenomenex), Thierry Domenger (Thermo) and Pilar Franco (Chiral Technologies) for the kind gift of columns.

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Figure captions

Fig. 1. Partial structures of the API and oxidation products examined in this study.

Fig. 2. Linear Solvation Energy Relationships (LSER) classification showing the 15 tested columns (achiral stationary phases in blue and enantioselective stationary phases in purple).

Fig. 3. Chromatogram of the sample from forced degradation obtained on Acquity Torus 2-PIC column (100 x 3.0 mm, 1.7 μ m) with the optimal elution gradient conditions as defined by the DoE: 100% MeOH co-solvent + 20mM AA + 2% water in mobile phase; gradient elution as indicated in the figure. UV detection 210 nm. Red star is the API (M); blue diamonds are the two isomeric mono-oxide species (M+16); green circle is the di-oxidized degradation product (M+32).

Fig. 4. Iso-response graphs of separation criterion S for the critical pair of peaks (peaks 3 and 4). The crossing of the dashed black lines represents optimal values of the DoE.

Fig. 5. Chromatogram of the sample from forced degradation obtained on Acquity Torus 2-PIC column (100 x 3.0 mm, 1.7 μ m) with optimal elution gradient and modified mobile phase composition: co-solvent 70%MeOH / 30% iPrOH comprising 20 mM AA + 2% water in mobile phase; gradient elution as indicated in the figure. UV detection 210 nm. Red star is the API (M); blue diamonds are the two isomeric mono-oxide species (M+16); green circle is the di-oxidized degradation product (M+32).

Fig. 6. Fraction collection (purple areas) in preparative supercritical fluid chromatography with elution gradient profile (green line). Separation done with Torus 2-PIC column (150 x 30 mm, 5 μ m) and mobile phase co-solvent comprising 70% MeOH and 30% iPrOH comprising 2% water and 20 mM ammonium acetate. UV detection 210 nm.

549 **Table 1**

550 The 15 columns used to screen the samples in this study

Column Name	Manufacturer	Particle type	Bonded ligand	Dimensions (mm)	Particle size (µm)
ACQUITY UPC ² Torus 2-PIC	Waters	Fully porous hybrid silica	2 Picolyl-amine	100 x 3.0	1.7
ACQUITY UPC ² HSS C18 SB	Waters	Fully porous high strength silica	Octadecyl, non endcapped	100 x 3.0	1.8
Kinetex HILIC	Phenomenex	Superficially porous silica	-	150 x 4.6	2.6
Accucore HILIC	Thermo	Superficially porous silica	-	150 x 4.6	2.6
Accucore Amide-HILIC	Thermo	Superficially porous silica	-	150 x 4.6	2.6
Accucore Urea-HILIC	Thermo	Superficially porous silica	-	150 x 4.6	2.6
Ascentis Express OH5	Supelco	Superficially porous silica	Penta-hydroxyl	150 x 4.6	2.7
Ascentis Express F5	Supelco	Superficially porous silica	Penta-fluorophenyl	150 x 4.6	2.7
Nucleoshell HILIC	Macherey-Nagel	Superficially porous silica	Sulfobetaine	150 x 3.0	2.7
Chiralpak IA	Chiral Technologies	Fully porous silica	Amylose <i>tris</i> -(3,5-dimethylphenylcarbamate)	150 x 4.6	3.0
Chiralpak IB	Chiral Technologies	Fully porous silica	Cellulose <i>tris</i> -(3,5-dimethylphenylcarbamate)	150 x 4.6	3.0
Chiralpak IC	Chiral Technologies	Fully porous silica	Cellulose <i>tris</i> -(3,5-dichlorophenylcarbamate)	150 x 4.6	5.0
Chiralpak ID	Chiral Technologies	Fully porous silica	Amylose <i>tris</i> -(3-chlorophenylcarbamate)	150 x 4.6	5.0
Chiralpak IF	Chiral Technologies	Fully porous silica	Amylose <i>tris</i> -(3-chloro-4-methylphenylcarbamate)	150 x 4.6	3.0
Chiralpak IG	Chiral Technologies	Fully porous silica	Amylose <i>tris</i> -(3-chloro-5-methylphenylcarbamate)	150 x 4.6	3.0

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Table 2

Design of experiments

	Final solvent percentage	Gradient time	Oven temperature
-α	60 %	3.0 min	20°C
-1	64 %	3.8 min	24°C
0	70 %	5.0 min	30°C
+1	76 %	6.2 min	36°C
+α	80 %	7.0 min	40°C

Table 3

Fractions analysis: collected masses and volumes, quality control for each fraction

	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Collected volume (L)	3.5	4.3	5.0	14.8
Collected mass (g)	ND	0.3	0.3	3
Fractions purity				
API	98.5 %	0.8%	ND	ND
Mono oxide 1	<1	95.8 %	0.1 %	ND
Mono oxide 2	<1	ND	97.5 %	ND
Di oxide	ND	2.6 %	2.1 %	99.3 %

*ND = Not determined

Fig. 1

★ API:
M ~ 900 Da

◆ Mono oxidized
product 1: M+16

◆ Mono oxidized
product 2: M+16

● Di oxidized
product : M+32

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Supercritical fluid chromatography

Design of experiments

Method optimization

Scale - up