DEVELOPMENT OF A HPLC/DAD ANALYTICAL METHOD FOR QUANTITATIVE DETERMINATION OF CLADRIBINE USING DESIGN OF EXPERIMENT

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INTRODUCTION

Cladribine (2-chloro-2'deoxyadenosine [CdA]) is a synthetic purine analogue, orally submitted for the treatment of relapsing-remitting multiple sclerosis[1]. Comparing to tablet formulations currently on the market (i.e. Mavenclad(R), Merck), innovative dosage forms such as **Orally Disintegrating Films (ODFs)** may provide better Cladribine release profiles and subsequential therapeutic advantages.
The aim of this study was to obtain a highly sensitive HPLC/DAD analytical procedure to use during development of a 10mg/dose Cladribine ODF in order to assay potency and assess in vitro dissolution of the API from the FDF.

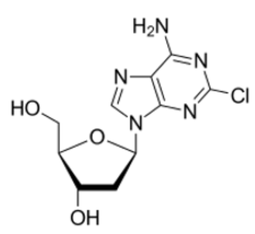

Figure 1: Structure of Cladribine (2-chloro-2'deoxyadenosine [CdA])

MATERIAL AND METHODS

Instrument:
Analytical development was carried out on Agilent 1260 Infinity II series HPLC system equipped with binary pump, autosampler, thermostated column compartment and Diode-Array detector (DAD), Agilent Technologies (Santa Clara, California, USA).

Solution preparation:
The solutions used for method development were prepared as follows:

- Solvent mixture: acetonitrile, water (10:90 V/V).
- Cladribine CRS stock solution (a): weigh 25,0±0,5 mg of Cladribine CRS (EDQM) into a 5 mL volumetric flask and dissolve in the solvent mixture using a heated ultrasound bath. Add the solvent mixture to volume and mix. This solution contains 5.0 mg/mL of Cladribine.
- Cladribine CRS stock solution (b): dilute 1.0 mL of Cladribine CRS stock solution (a) to 100.0 mL with the solvent mixture. This solution contains 50 µg/mL of Cladribine.
- Cladribine DoE solution: dissolve 1.0 mg of Cladribine impurity C CRS (EDQM) in 25 mL of Cladribine CRS stock solution (b) and dilute to 50.0 mL with solvent mixture. This solution contains about 25 µg/mL of Cladribine and 20 µg/mL of impurity C.

DoE software:
Design-Expert, version 13.0, Stat-Ease Inc. (Minneapolis, Minnesota, USA)

EXPERIMENTAL

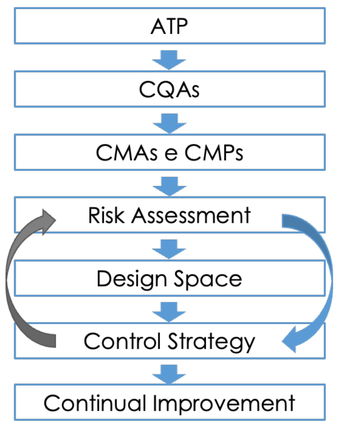

Figure 2: Analytical method development workflow

Analytical method development workflow (**Figure 2**) followed the new ICH Q14 [2] undertaking a Quality-by-Design (QbD) approach implying Design of Experiment (DoE):

I - definition of the Analytical Target Profile(ATP) and the Critical Quality Attributes (CQAs) of the analytical method
II - definition of the Critical Material Attributes (CMAs) and Critical Method Parameters (CMPs) and their associated risk of affecting CQAs, through application of quality risk assessment tools (i.e. FMECA, check lists, DoE)
III - Application of Design of Experiment (DoE): a pre-planned series of analysis, with a systematic variation of controllable experimental factors (CMPs) that induce a response in a system, was performed in order to obtain a mathematic factor-response correlation model
IV - Risk control: the mathematic correlations were analyzed, and the analytical method was modified in order mitigate or even eliminate risks on CQAs. Reiteration of points II-IV allowed optimization of the analytical method within three steps (**Table I**).
V - Continuous monitoring and updating of the analytical procedure to ensure reproducible quality

Analytical target profile:The resulting method should be able to quantify the API in the presence of possible relevant impurities, within a range of 10-125% of the nominal concentration, in a 500mL vessel, with precision and accuracy according to ICH Q2(R2). Total runtime analysis should be under 15 minutes.
Cladribine stock solution(a) was kept at different storage conditions (7°C, 25°C and 40°C) in order to assess stability and possible degradation products: the solution was sampled and tested for related substances by HPLC/UV using the compendial Ph. Eur. method at different time points. During this study, it was found that Cladribine's main thermal degradation product is 2-Chloroadenine, listed in EP with the name "Cladribine Impurity C" [Imp C]. Seen the use of high temperatures in the manufacturing process of Cladribine ODFs currently under development, Cladribine's resolution from this particular degradation product was selected as a CQA for analytical development.

	Primary parameter screening	Secondary parameter screening	Method optimization
Design	/	two-level fractional factorial	Face-centred CCD RSM
Runs	20	39	36
CMPs (Levels)	Injection volum (20µL) Wavelenght (265nm) Stationary Phase: Luna C8 25cm x 4.6mm x 5µm Mobile Phase A: water Mobile Phase B: ACN Time before gradient (1 min) Time on analysis (15 min)	A: % H3PO4 (0-0.35% V/V) B: column oven (25-45°C) C: flow (0.6-1.2 mL/min) D: % B at beginning (0-20%) E: % B at the end (40-60%) F: gradient time (4-6min)	A: flow (0.6-1.2 mL/min) B: % B at beginning (0-20%) C: % B at the end (40-60%)
CQAs	W: width(s) H: height(mAU) A: area (mAU*s) Rt: retention time NTP: number of theoretical Plates Tf: tailing factor Rs: resolution between CdA and Imp C	H: height(mAU) Rt: retention time Tf: tailing factor Rs: resolution between CdA and Imp C	H: height(mAU) Rt: retention time

Table 1: Summary of Experimental Phases and Factors and Responses investigated

Moreover, several peak shape and performance parameters were monitored as to guarantee chromatographic separation suitability and method sensitivity (concentration range for Cladribine quantitation was set between 1-15µg/mL).
Cladribine is insoluble in acetonitrile, slightly soluble in methanol and water[3], in which dissolution is slow and requires the use of heated ultrasound bath. Scarce solubility in the solvent mixture and mobile phases resulted in longer sample preparation time, but was not considered a CMA.

A **preliminary parameter screening,** allowed to choose basic method criteria: acetonitrile[4] was preferred to methanol[5], in the role of organic mobile phase modifier, since it provided a more efficient elution without affecting solubility of the API. A Luna C8 column (25cm x 4.6mm - 5µm porosity) was selected, the same stationary phase as related substances assay. Lastly, an injection volume of 20 µL and detection at 265 nm were selected in order to ensure good sensitivity of the analytical method.
From the results of preliminary screening and prior knowledge of chromatography, a brainstorming session was conducted for the identification of Critical Method Parameters (CMPs) and their corresponding **risk assessment**: risk priority number (RPN) was calculated by the multiplication of scores of occurrence, severity and detectability for each CMP on CQAs and entered in an excel sheet of CQAs against the respective potential method risk parameter.
A **secondary parameter screening,** employing a two-level fractional factorial design, was done to investigate the correlations between the CMPs and responses.The column oven temperature modifications resulted in non-significant correlations. The best gradient time was 6 minutes. It was found that the amount of orthophosphoric acid (A) in the mobile phase, has non-significant correlations with all the CQAs investigated, except Cladribine's peak tailing factor and resolution from Imp C. Cladribine tailing factor, resulted between 0,8 and 1,5 for all analytical conditions examined, complying with Ph. Eur. requirements. The mobile phase acidity was hence set at 0,085% v/v orthophosphoric acid, since it was found that by setting a gradient time of 6 minutes, column oven temperature at 25°C and a 0,085% v/v of orthophosphoric acid, for any value of flow rate (C) and % of mobile phase B at the beginning of gradient (D), Rs>1,2 as shown in the contour plots (**Figure 3**). The following **risk control**, identified risk reduction for Tf and Rs responses that were no longer considered CQAs.

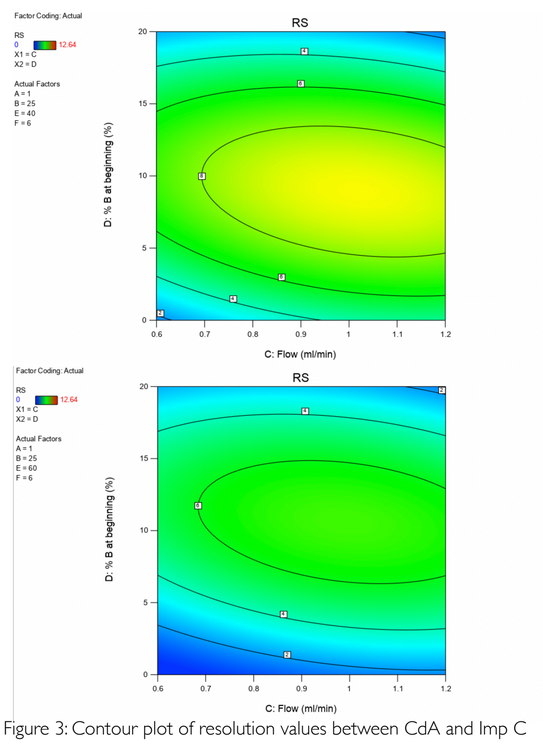

Figure 3: Contour plot of resolution values between CdA and Imp C

Lastly, a face-centered central composite design response surface method (face-centered CCD RSM) was employed to achieve **method optimization** for the remaining three factors. For all responses, it was selected a mathematic model of quadratic order with R^2 >0.98. Maximization of peak height, with a minimal value of 400 mAU in order to achieve the needed analytical sensitivity and a retention time between 6 and 8 minutes were selected as desirability critera in the optimization tool. Among the several solutions with decreasing desirability generated by the software, was chosen the one with higher desirability and round operation parameters (**Figure 4**) . The predicted responses of the selected solution were then confirmed in triplicate analysis.

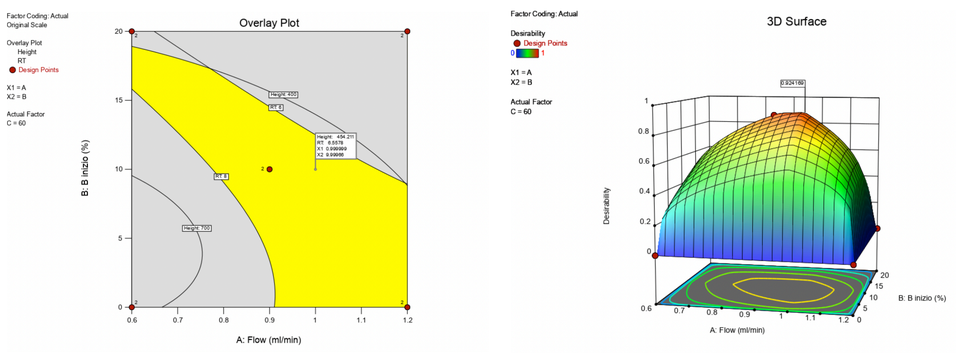

Figure 4: chosen method conditions position in the Design Space (overlay plot - right) and their desirability (3D surface - left)

CONCLUSION

A simple, swift and sensitive HPLC/DAD method for the determination of Cladribine in ODFs was developed using a QbD approach and DoE . The optimized method conditions are listed in **Table II,** while a resulting chromatogram example is shown in **Figure 5.**

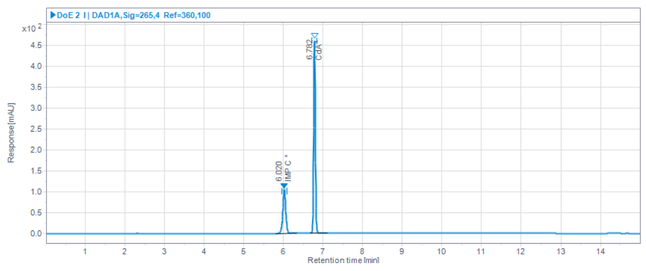

Figure 5: Chromatogram of a standard mix solution containing 25 µg/ml of Cladribine and 20 µg/ml of impurity C at the optimized method conditions

Injection volume	20µL		
Column	Luna C8(2) 25cm x 4,6mm, 5µm ,100Å (Phenomenex)		
Oven Temperature	25°C		
Mobile phase A	Water MilliQ + H_3PO_4 conc. for HPLC 0,085% v/v		
Mobile phase B	ACN + H_3PO_4 conc. for HPLC 0,085% v/v		
Flow Rate	1 mL/min		
Gradient	Time (min)	A (% v/v)	B (% v/v)
	0	90	10
	1	90	10
	7	40	60
	9	40	60
	10	90	10
	15	90	10
Run Time	15 minutes		

Table II: Summary of optimized analytical method conditions

References:

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[2] International Council for Harmonization of technical requirements for pharmaceuticals for human use, ICH harmonize Guideline "Analytical procedure development" Q14, draft version, endorsed on 24 March 2022, currently under public consultation
[3] Ph. Eur.10.0, Cladribine monograph 01/2011:2174
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[5] United States Pharmacopeial Convention Committee of Revision (Ed.), *USP-NF Online* (44th ed.). United States Pharmacopeial Convention, Cladribine monograph