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Computer-assisted UHPLC method development and optimization for the determination of albendazole and its related substances

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ABSTRACT

Computer-aided ultrahigh performance liquid chromatographic (UHPLC) method development and optimization was undertaken in order to replace an underperforming European Pharmacopoeia method for the determination of albendazole and its related substances. In the preliminary screening, a temperature-gradient time bidimensional model was chosen to aid selection of the proper stationary phase. Hereinafter temperature-gradient time-ternary composition and temperature-gradient time-pH tridimensional models were applied for the optimization of critical method parameters. The simulation and *in silico* robustness testing were realized using DryLab modeling software. The final method was validated for quantification of impurities and assay of the active substance according to the current ICH guidance. The validated methods were tested on a real, commercial tablet formulation. The experimental design-based and software-assisted method development proved to be a fast and reliable way of replacing a method with inadequate selectivity and long runtime with a robust UHPLC-based method, which offers baseline separation for all monitored impurities in 10 min. Results confirm that software-based chromatographic modelling can not only speed up the analytical method development process, but also improve the reliability of the developed method.

1. Introduction

Quality by design (QbD) is a new, but already widely implemented knowledge-oriented trend in pharmaceutical production. The International Council on Harmonization (ICH) defines QbD 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*" [1]. The focus of this concept is that the quality should be built into the product, by a systematic understanding of the process, rather than relying on finished product testing. Details regarding the application of the QbD concept for product development is highly detailed in the ICH Q8-Q11 guidelines [1–4]. Following the example of successful application of QbD principles in pharmaceutical manufacturing, a similar paradigm shift is also underway in the development of analytical methods. Analytical Quality by Design (AQbD) is a scientific-based concept of method development, using carefully designed experiments and computer-aided statistical interpretation and modeling to obtain optimal method performance. Starting from the predefined objectives described in the Analytical Target Profile (ATP), the aim of this concept is to identify correlations between the critical method parameters (CMPs) and critical method attributes (CMAs), through multivariate experimental design and analysis. In this way an appropriate level of method understanding can be achieved and operating region can then be defined where there is high confidence that the method will operate reliably. Although currently there is no official guideline for the implementation of AQbD, only the ICH Q14 initiative [5], a few technical reviews [6–13], and in addition, several scientific papers have recently been published highlighting the advantages and applicability of AQbD principles [12–18].

The traditional way of chromatographic method development is based on the empirical trial-and-error approach, which often results in a lack of adequate process understanding and unforeseen difficulties during method validation. Design of experiments (DoE)

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on the other hand offers advantages and can be done using statistical analysis software (Modde (Umetrics, Sweden), Design-Expert (Stat-Ease, Minneapolis, USA)) or with elution prediction based on the chemical structure of the analytes (EluEx (CompuDrug Chemistry, Hungary)). Another alternative is retention modeling based on planned preliminary runs (ChromSwordAuto (Dr Galushko Software, Germany), ACD/AutoChrom (ACD/Labs, Toronto, USA)), or DryLab (Molnar Institute, Germany)). Some of these software allow the full analytical lifecycle management of the chromatographic method from optimization to auditing (Fusion (S-Matrix, Indianapolis, USA)). The basics of chromatographic retention modelling were established with the creation of the solvophobic theory by Csaba Horváth [19]. This concept was put into practice in the 1980's, when Lloyd Snyder and his workgroup established the first One-Factor-At-A-Time (OFAT) retention modeling software (DryLab) for isocratic and gradient elution [20,21]. This was followed by the two-dimensional model using the DryLab software, when the effect of two factors (temperature and gradient) was investigated at the same time [22], allowing in-depth characterization of stationary phases also [23,24]. Nevertheless, the big breakthrough was the three-dimensional experimental model [25]. In this case using only 12 experimental runs the modeling software established the "Cube", a 3D critical resolution map as a function of the critical method parameters (CMPs) and attributes (CMAs). This approach allowed the simultaneous optimization of three factors with a high level of process understanding. The general workflow of the systematic analytical method development, using DryLab, was described in previous papers [26,27].

Another crucial point of a chromatographic method is its robustness or ability of the method to tolerate the changes in parameters or equipment, without a negative impact on the quality of the delivered result [28]. Classical robustness testing, *i.e. c*hanging one variable at a time and measuring its impact on separation performance and results, is usually performed during late method development or method validation. This approach can be extensively time- and resource consuming. Moreover, method weaknesses are usually discovered only in routine use and can significantly shorten the lifetime of a method. In contrast, computer-assisted elution modelling allows the *in silico* verification of the sensitivity of the method to variations. With the simulation of all the combinations of the process parameters, using a multivariate approach, the robust area of the resolution map is visualized and the effect of the individual factors and the correlation between them are predicted [26,29,30]. To ensure that the final method fulfills the expectations during its lifecycle, the use of a control strategy is recommended, with periodical verification of system suitability criteria.

Albendazole is an oral anthelmintic drug used for the treatment of a variety of parasitic worm infestations, like giardiasis, trichuriasis or ascariasis. A literature survey reveals that Gomes and Nagaraju described a liquid chromatographic method for the determination of process related impurities of anthelmintic drugs. In case of albendazole, a mobile phase of methanol:water:perchloric acid 45:55:0.3 (v/v/v) was used and separations were performed on a C18 column, under isocratic mode. However, the authors monitored only two related substances of albendazole (albendazole sulphone and albendazole sulphoxide) in bulk drugs [31]. The active substance is also official in the 10th edition of European Pharmacopoeia (Ph. Eur. 10th) [32]. However, the HPLC method described in the monograph is inadequate for the simultaneous quantification of all individual impurities since it does not provide baseline resolution of all peaks of interest. Physico-chemical properties of these degradation products, like dissociation constant (pKa) or partition coefficient (logP) are similar, therefore the identification of optimal separation conditions can be challenging for analysts. However, by using the opportunities provided by computer-aided retention modelling, the development and optimization processes can be accelerated, and the suitability of the final method can be ensured.

Starting from these presumptions, the aim of our study was to develop an UHPLC method which is capable for the simultaneous separation and quantification of albendazole and its impurities, ensuring adequate selectivity within a short analysis time. To meet the regulatory authority's requirements, the workflow of a systematic method development based on AQbD principles was followed. The experimental design framework, construction, and interpretation of virtual separation models and the *in silico* robustness testing were realized using the DryLab 4 software. The validation of the optimized method in accordance with ICH principles is also the subject of this study.

2. Experimental

2.1. Chemicals and samples

2.1.1. Chemicals

The aqueous part of the mobile phase in this study was a 10 mM aqueous sodium acetate solution with various pH values. The HPLC grade anhydrous sodium acetate salt (with purity $\geq$ 99.0 %) used for the preparation of buffer solution and the chromatographic grade glacial acetic acid (with purity $\geq$ 99.7 %) used for pH adjustment of the buffer solutions were ordered from Merck (Darmstadt, Germany). Gradient grade methanol (MeOH) and acetonitirile (ACN) were purchased from Merck (Darmstadt, Germany). Ultrapure water was freshly prepared each day by a MilliPore MilliQ Integral 10 (Merck Millipore, USA) equipment. Albendazole and its related substances (A, B, C, D, E and F) were from LGC Standards (Teddington, London, United Kingdom). The chemical structures, IUPAC names and the physical-chemical properties (log P and pKa) of the analytes are shown in Fig. 1.

All buffer solutions were filtered using membrane filter (Millipore Durapure with 0.22 μm pore size, ordered from Merck, Darmstadt, Germany) with the aid of a vacuum pump and degassed in an ultrasonic bath.

2.1.2. Sample solutions

The solvent used for sample preparation was gradient grade MeOH with 1 % (v/v) glacial acetic acid, added to lower the pH and to improve the solubility of albendazole [33]. All the sample solutions were filtered using Whatman Puradisc PTFE syringe driven filter units (Merck, Darmstadt, Germany) with 0.20 μm pore size, to eliminate the potential insoluble particles.

2.1.3. Artificial samples for retention modelling

The sample used for the software-aided retention modelling was an artificial mixture containing 100 μg/mL albendazole spiked with all impurities, in the following concentrations: impurity A 9 μg/mL, impurity B 6 μg/mL, impurity C 7 μg/mL, impurity D 4 μg/mL, impurity E 5 μg/mL and impurity F 11 μg/mL. The purpose of applying different concentrations was to facilitate peak tracking in different chromatographic conditions.

2.1.4. Real samples analysis

The optimized method was applied on real commercial samples, acquired from a local pharmacy in Targu Mures. The samples were uncoated tablets, labelled to contain 200 mg albendazole. 20 tablets were individually weighted, and the average tablet weight was determined. Sample solutions were prepared as follows: ten tablets were manually powdered with a pestle in a porcelain mortar and an amount of tablet powder corresponding to 50 mg albendazole was measured into a 100 mL volumetric flask, 30 mL solvent was added, and the mixture was sonicated for 15 min using an ultrasonic bath and completed with solvent, afterwards. This resulted in a final

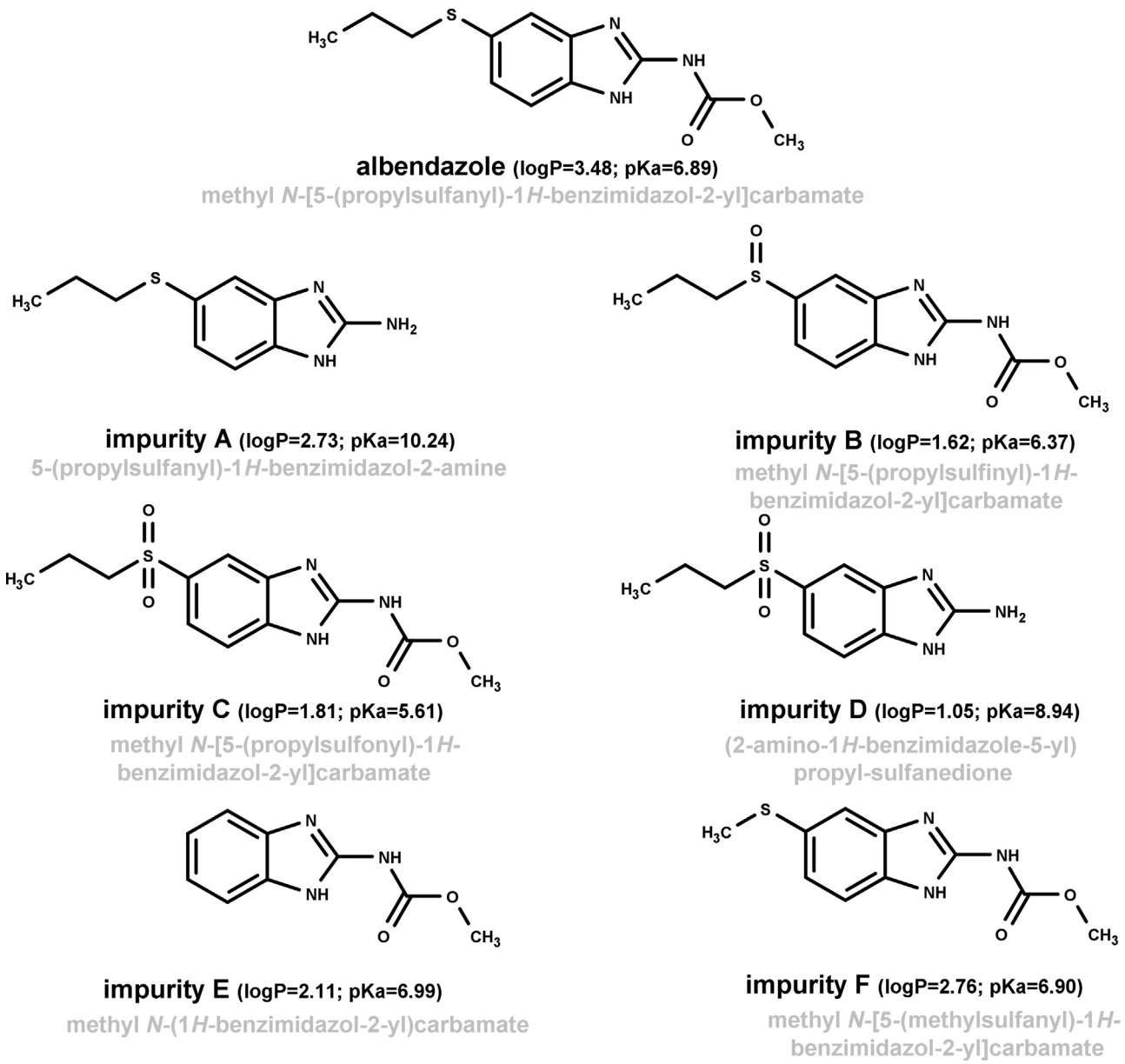

Fig. 1. The chemical structure, IUPAC names and estimated logP and pKa values of the analytes.

theoretical concentration of 500 μg/mL albendazole. All impurity levels expressed in percentages are reported to this concentration.

The method was also validated and applied for the assay of the active substance. In this case, 4 mL of the abovementioned sample solution was diluted to 10 mL in a volumetric flask with solvent. This resulted in a final theoretical concentration of 200 μg/mL albendazole.

2.1.5. Samples for method validation

The optimized method was validated for both the impurity testing and assay of albendazole from tablet dosage form.

To prove the linearity of the impurity method, a stock solution was prepared, containing albendazole and all impurities in 10 μg/mL concentration, and this solution was diluted at 12 levels in the range of 0.10–10 μg/ml (0.02–2.0 %, relative to the active substance concentration used in impurity testing). For the linearity of the assay method 500 μg/mL albendazole stock solution was prepared and diluted at 11 levels in the range of 100–500 μg/ml (50−250 %, relative to the test concentration used in assay).

The accuracy of the impurity method was tested by adding known amount of all impurities to the placebo powder at four levels (level 1: 0.5 μg/mL (0.1 %); level 2: 1 μg/mL (0.2 %); level 3: 3 μg/mL (0.6 %) and level 4: 7.5 μg/mL (1.5 %)), covering the linearity range, with three replicates at each level, and the precision was tested using real impurity test solutions spiked with a known amount of impurities, within the linearity range. The spiking was performed, because not all impurities were present in the normal sample solution. The accuracy of the assay method was tested at three levels (level 1: 160 μg/mL (80 %); level 2: 200 μg/mL (100 %); level 3: 220 μg/mL (120 %)), with three replicates on each level, using samples prepared from albendazole reference substance with known purity, in the linearity range. The precision of the assay method was tested using test solutions with 200 μg/mL albendazole content. To prove the precision of the impurity method, an artificial mixture was used containing 500 μg/mL albendazole and 3 μg/mL of all impurities.

2.2. Equipment and software

The analytical balance (MT XPE 205) and pH-meter (SEVEN EXC MT) used during the experiments were purchased from Mettler-Toledo (Mettler-Toledo, Greifensee, Switzerland). The ultrasonic bath (Elmasonic P180 H) and the vacuum pump (KNF Laboport) used in the process of preparing the sample and buffer solutions were ordered from Elma Schmidbauer (Singen, Germany) and Merck, respectively (Darmstadt, Germany)

The chemical structure of the molecules and the physical-chemical parameter estimation of the compounds were realized using the demo version of MarvinSketch software (ChemAxon, Budapest, Hungary).

UHPLC experiments were performed on an Agilent Infinity 1290 chromatographic system (Santa Clara, California, USA) equipped with quaternary solvent delivery pump (G4204A), autosampler (G4226A), autosampler thermostat (G1330B), column thermostat (G0316C) and photodiode array detector (G4212A). The dwell volume of the system was measured as 450 μL. All chromatographic data were acquired and processed by OpenLAB (EZChrom Edition) software (Agilent, Santa Clara, California, USA). After integration, the chromatograms were exported into AIA/ANDI-format (*.cdf) (including retention times and peak areas) and were directly imported to the modelling software for further processing and peak tracking. The experimental design and interpretation of the obtained data, including subsequent method optimization and *in silico* robustness testing were realized using DryLab 4 (version no 4.3.5.4) chromatographic retention modeling software (Molnár-Institute, Berlin, Germany).

Drylab as a software can handle the simultaneous variation of maximum three experimental factors, using *tG-T-pH* or *tG-T-tC* designs and twelve chromatographic runs. In the current software version, the simultaneous variation of both pH and ternary composition is not available. However, such designs were explored by the software creators and applied successfully [29], suggesting that future versions of Drylab will most likely include these designs also. The impact of instrumental factors (dwell volume, column parameters, flow rate, column length, diameter etc.) can also be visualized, allowing a broader flexibility.

2.3. HPLC columns

Three different columns were used during the method development, with similar dimensions for better comparability: Zorbax Eclipse Plus C18 Rapid Resolution High Definition (50 × 2.1 mm, 1.8 μm) with fully porous silica particles (Agilent, Santa Clara, California, USA), Poroshell 120 EC C18 (50 × 2.1 mm, 2.7 μm) with core-shell type silica particles (Agilent, Santa Clara, California, USA) and ACE Excel C18-PFP (50 × 2.1 mm, 1.7 μm) with fully porous silica particles (Advanced Chromatography Technologies, Aberdeen, Scotland).

The method described in the Ph. Eur. 10th monograph [32] specifies a 250 × 4.6 mm C18 column with 5 μm average diameter particles, therefore the original method was tested using a Purospher RP-18 endcapped (250 × 4.6 mm, 5 μm) column (Merck, Darmstadt, Germany).

2.4. Design of experiments

To identify the most suitable stationary phase with the highest selectivity for the tested analytes, a systematic preliminary scouting process was realized. For the sake of objective comparison, the same bidimensional (2D) gradient time (*tG*) and temperature (*T*) experimental framework (*tG-T,* Fig. 2A) was realized using all three selected columns. Experiments were performed at two levels, whereas the difference between the short and long gradient time was as a factor of three (tG_1 = 5 min and tG_2 = 15 min, from 5 % to 70 % organic solvent) and in the case of the temperature was 30 °C (T_1 = 20 °C and T_2 = 50 °C). This 2D experimental design needs 4 (2 factors at 2 levels, 2^2 = 4) corner experiments for the construction of the retention model. The mobile phases were A: 10 mM aqueous sodium acetate buffer pH 4.2 and B: ACN.

After column selection, different three-dimensional (3D) experimental designs were also applied to optimize the chromatographic parameters, others than the stationary phase. Firstly, a gradient time-temperature-ternary composition (*tG-T-tC,* Fig. 2B) design was applied to evaluate the effect of the organic modifier. The ternary composition (*tC*) of the mobile phase was investigated at three levels using 100 % ACN (B1), 100 % MeOH (B2) and their 50:50 % (B1 + B2) mixture, with a gradient range from 5 to 80 % organic component. The aqueous part of the mobile phase was acetate buffer solution with pH 4.5. Secondly, the impact of pH of the aqueous part of the mobile phase was studied using a similar 3D gradient time-temperature-pH model *(tG-T-pH,* Fig. 2C). The effect of pH of the sodium acetate buffer solution was investigated at three levels (pH_1 = 3.6, pH_2 = 4.2 and pH_3 = 4.8) and ACN was used as organic solvent with a linear gradient from 5 to 70 %. In both cases the gradient time and the temperature were fixed at two levels (tG_1 = 5 min, tG_2 = 15 min, T_1 = 20 °C, T_2 = 50 °C) and the construction of the models needed 12 corner experiment (2 factors at 2 levels and 1 factor at 3 levels 2^2 x 3^1 = 12). The structure of the experimental designs (DoE) is represented on Fig. 2.

The parameters and the differences between the levels were selected based on the physical-chemical properties of the analytes (Fig. 1), previously published similar studies [34,35], and also taking into consideration the recommendations of the DryLab software. In all the experiments mentioned above, the flowrate was 0.5 mL/minute, and the injection volume was 1 μL, while detection was performed at 254 nm.

Since the final method must be capable of producing adequate results for an extended period, in complex analytical environments, *in silico* testing was performed to evaluate method robustness, using DryLab 4.

3. Results and discussion

3.1. Preliminary experiments

Upon application of the Ph. Eur. 10th method (Supplementary Table 1) described for the determination of related substances of albendazole [32] several shortcomings were identified, which rendered it unfit for routine use. Impurities B and C co-eluted, and baseline separation between impurities E and A was also not achieved (Supplementary Fig. 1). One of the potential failure sources could be the lack of pH control of the mobile phase, because analyte ionization and implicitly chromatographic retention of these compounds with different pKa values (Fig. 1) is highly influenced by mobile phase pH. Considering the chromatographic behavior of the analytes and the logP values, lipophilicity of the molecules varies widely, therefore gradient elution could also be more advantageous than the applied isocratic conditions described in Ph. Eur. 10th monograph.

Due to the abovementioned aspects, the development of a new UHPLC method was decided. The method should be capable not only of the determination of related substances of albendazole, but also the assay of the anthelmintic drug in tablet formulations. During routine impurity analysis, probably proper selectivity and total run time are the most critical factors; therefore, the main objective of this research was to achieve a robust method, which can provide baseline separation of all analytes, ($R_{s,\,crit.} \geq 1.5$) in a short runtime (total run time < 10 min). In recent years, there is an increase

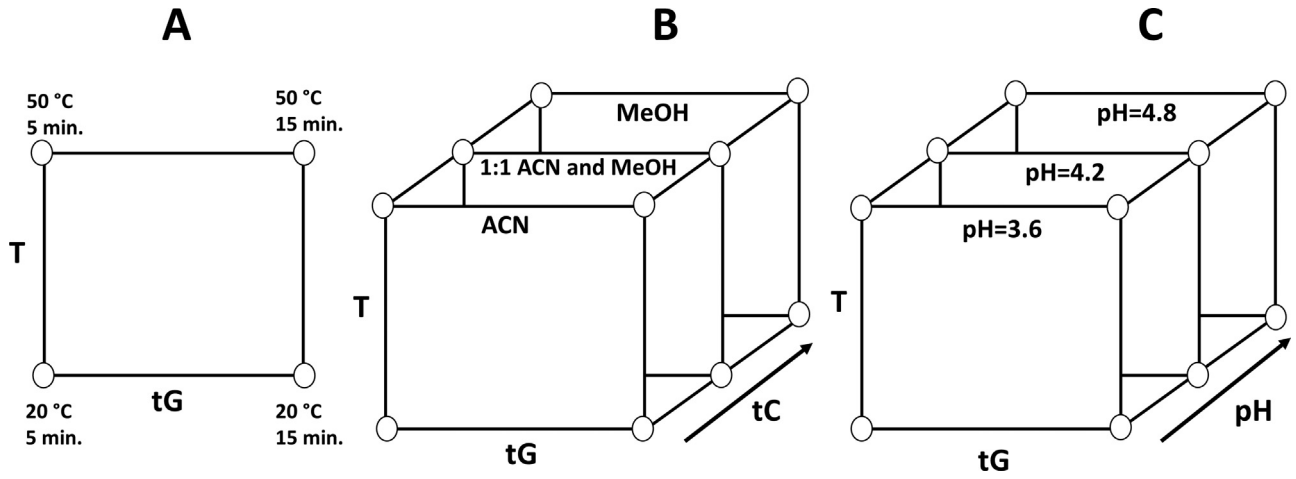

Fig. 2. The experimental designs used during method development: (**A)** 2D *tG-T* model; (**B)** 3D *tG-T-tC* model; (**C)** 3D *tG-T-pH* model.

in demand for combined methods which could be applicable for both assay of the active substance and determination of its related substances [36,37]. These methods could further increase throughput in routine quality control labs. Thus, a secondary objective was also to test whether the same method can be applied in both cases, for tablets containing albendazole.

3.2. Column selection

Corresponding to the *tG-T* experimental design the same four corner runs were performed on three HPLC columns with the same dimensions (Zorbax Eclipse Plus C18 Rapid Resolution High Definition 50 × 2.1 mm, 1.8 μm; Poroshell 120 EC C18 50 × 2.1 mm, 2.7 μm and ACE Excel C18-PFP 50 × 2.1 mm, 1.7 μm). The obtained chromatograms provided the input data for the DryLab software to construct the bidimensional resolution maps (Supplementary Fig. 2), which are the visual representations of the critical resolutions in function of the selected method parameters. The part of the resolution map where the method meets the preset critical resolution value is highlighted with red. To prove the validity of the obtained virtual separation models, two setpoints - with the highest predicted resolution of critical peak pairs - were selected and verified, by comparing the theoretically predicted and experimentally obtained chromatograms (comparative data in form of retention times and resolutions are summarized in Table 1). The correlation between the predicted and experimentally obtained retention times was investigated plotting the retention times estimated by the software, against the experimentally obtained values and calculating the differences (average of retention time errors % percentages) and coefficient of determination (R^2) between these values. This can be realized automatically, using the "Confirmation run" function of the DryLab software. The main consideration for column selection in evaluating the obtained results was the maximum achievable resolution between critical peak pairs.

The Zorbax Eclipse Plus C18 column with fully porous endcapped octadecylsilyl modified particles was selected due to the similar chemistry to the recommended column of the Ph. Eur. 10th monograph. In this case the critical peak pairs were the impurities C and A, and the best achieved experimental resolution value was 4.43, with setpoint 1 (Supplementary Fig. 3).

The Poroshell 120 EC C18 stationary phase theoretically is equivalent with the former, regarding to their chromatographic behavior. Due to the core-shell particle type, backpressure was significantly lower on this column, which could be an advantage in routine use, however the maximum achievable experimental $R_{s,\,crit.}$ value was 1.66 in setpoint 1 (Supplementary Fig. 4), which is much lower than that obtained on the former column. The software can also predict the retention times with other flowrates in addition to the 0.5 mL/min used in the input experiments. At setpoint 2 a 0.7 mL/min flow was tested and, in this case, as well the correlation between the experimental and predicted retention times was particularly good.

Due to its similar hydrophobic characteristics, ACE Excel C18-PFP column may be used for applications in which "standard" C18 columns would normally be considered or can be used to improve separations that are proving to be problematic on C18 columns. In many instances, the same conditions that prove unsuitable for the C18 column can be appropriate for the C18-PFP column, therefore, we also selected this column in the study. Based on the resolution map the setpoint 1 (Supplementary Fig. 5) with a very steep gradient is the most suitable condition, but the resolution between the impurities A and F is overestimated by software, and under the selected experimental conditions these peaks co-elute. Although the $R_{s,\,crit.}$ is overestimated, retention time prediction of the software is acceptable. In all three cases simulated results were found to be in excellent agreement with the experimental ones (average of retention time errors were 1.18% and 3.32%), proving the usefulness and effectiveness of retention modelling.

Based on Supplementary Figs. 3,4 and 5, under the applied conditions, peak efficiencies and asymmetry values were slightly better on the Zorbax Eclipse Plus C18 column, followed by the Poroshell 120 EC-C18 column. The lowest theoretical plates and highest overall asymmetry values were observed on the ACE Excel C18-PFP column. The highest $R_{s,\,crit.}$ was also obtained on the Zorbax Eclipse Plus C18 column, therefore this was selected for further method optimization.

3.3. Method optimization

The effect of the organic modifier type was tested with a tridimensional *tG-T-tC* experimental design, where the organic part of the mobile phase (*tC*) was modified at three levels (100 % ACN (B1), 50:50 % mixture of ACN and MeOH (B1 + B2) and 100 % MeOH (B2), all percentages being expressed as v/v %). Based on previous experiments pH = 4.5 was selected, at this value the critical peak pair is impurities A and F. The obtained 3D resolution map is presented in Supplementary Fig. 6. To better visualize the effect of composition of the ternary mobile phase, three software simulated chromatograms are presented with increasing MeOH content in the mobile phase, in all cases at *tG* = 5 min, and *T* = 40 °C (Fig. 3). It is observable that low MeOH content (20 %) has no significant benefic effect on the critical resolution (Fig. 3B), compared to neat

Table 1
Summary of the predicted and experimentally obtained data during the column screening process.

Column	Zorbax Eclipse Plus C18				Poroshell EC 120 C18				ACE Excel C18-PFP			
Setpoint no.	Setpoint 1		Setpoint 2		Setpoint 1		Setpoint 2		Setpoint 1		Setpoint 2	
Conditions	tG = 8 min. (15–35 % ACN) T = 35 °C 0.5 mL/min.		tG = 8 min. (10–40 % ACN) T = 45 °C 0.5 mL/min.		tG = 4.5 min. (15–75% ACN) T = 28 °C 0.5 mL/min.		tG = 5 min. (15–70% ACN) T = 20 °C 0.7 mL/min.		tG = 3 min. (20–90% ACN) T = 17 °C 0.5 mL/min.		tG = 10 min. (10–70% ACN) T = 40 °C 0.5 mL/min.	
R_t (min.)	Drylab	Exp.	Drylab	Exp.	Drylab	Exp.	Drylab	Exp.	Drylab	Exp.	Drylab	Exp.
imp. D	0.68	0.69	1.27	1.17	0.65	0.67	0.52	0.52	0.66	0.64	1.67	1.55
imp. E	1.55	1.46	2.32	2.23	1.39	1.31	1.09	1.03	1.05	0.88	2.29	2.24
imp. B	1.91	1.79	2.96	2.94	1.55	1.48	1.22	1.19	1.21	1.09	2.51	2.49
imp. C	2.86	2.81	3.66	3.66	1.99	1.99	1.67	1.67	1.60	1.58	3.18	3.17
imp. A	3.20	3.28	3.97	4.01	2.19	2.24	1.87	1.92	1.79	1.89	3.74	3.78
imp. F	3.77	3.81	4.39	4.41	2.34	2.37	2.01	2.03	1.85	1.89	3.74	3.78
Albendazole	7.36	7.70	7.16	7.26	3.30	3.38	3.01	3.07	2.34	2.44	5.41	5.45
R^2	0.9989		0.9999		0.9970		0.9988		0.9875		0.9994	
Average of retention time errors (%)	0.76		1.49		0.28		0.40		3.32		1.18	
Rs crit.	4.08 (C-A)	4.43 (C-A)	2.67 (C-A)	2.72 (C-A) 1.91 (A–F)	1.83 (E-B) 1.66 (A–F)	1.88 (E-B) 1.89 (A–F)	1.82 (E-B) 1.50 (A–F)	1.90 (E-B)	1.17 (A–F)	0 (A–F)	0 (A–F)	0 (A–F)

(Drylab - retention time predicted by the DryLab software; Exp. – experimental obtained retention time; Retention time error (%) = (DryLab-Exp.)/Exp*100; R_t -retention time; $R_{s,\,crit.}$ - critical resolution; imp. – impurity; C-A – resolution between impurities C and A; E-B – resolution between impurities E and B; A–F – resolution between impurities A and F).

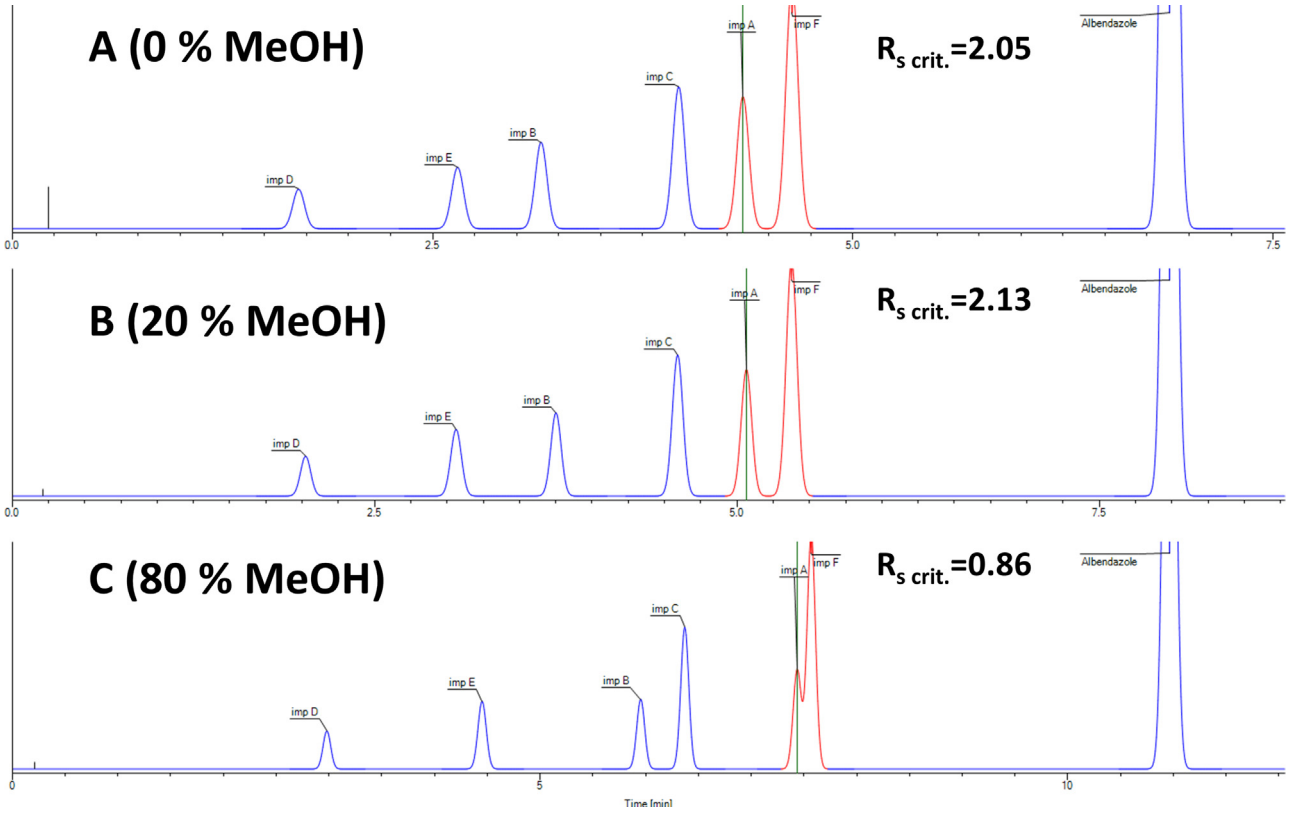

Fig. 3. The effect of ternary composition of the mobile phase on the critical resolution **(A)** 0 % MeOH in ACN, **(B)** 20 % MeOH in ACN and **(C)** 80 % MeOH in ACN.

ACN (Fig. 3A). At high MeOH composition the resolution between the impurities A and F decreases largely (Fig. 3C). According to this model, neat MeOH as the organic modifier is not providing the proper selectivity and using an ACN-MeOH mixture has no benefic effects on the resolution. A general major drawback of the MeOH-water mixture is the high backpressure generated in the chromatographic system, meaning disadvantage in routine use, regarding the apparatus and column lifetime, therefore ACN was selected as the sole organic modifier for further method optimizations.

To discover and understand the combined effects of pH, gradient time and temperature on the separation, a similar a 3D model was constructed, but instead of *tC* the *pH* of the aqueous phase was changed at three levels (pH_1 = 3.6, pH_2 = 4.2 and pH_3 = 4.8), in accordance with the *tG-T-pH* experimental design. Fig. 4A represents the obtained 3D resolution map, allowing the visualization of the influence of *tG*, *T* and *pH* on $R_{s,\,crit.}$ A minimum critical resolu-

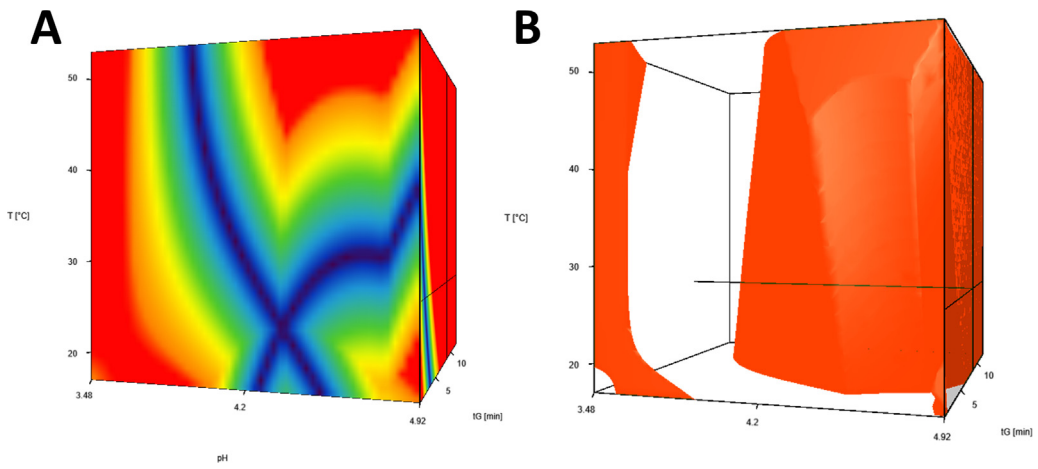

Fig. 4. **(A)** 3D resolution map of the obtained *tG-T-pH* model**, (B)** regions of the model, where $R_{s,\,crit.}$>1.5 criteria is met.

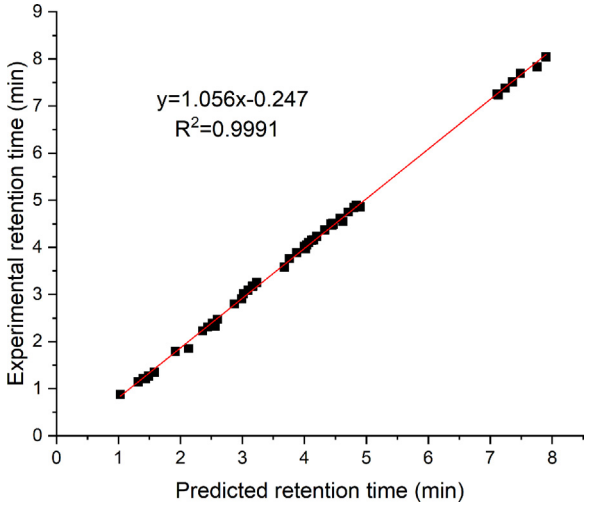

Fig. 5. Plot of experimental versus predicted retention times for all analytes obtained under the six different setpoints used for verification of the *tG-T-pH* model.

tion $R_{s,\,crit.} \geq 1.5$ was defined and red zones inside the cube (Fig. 4B) indicate 3D regions where this criterion is met. At lower pH values, almost no red zones can be observed, due to the coelution of impurities C and A, while in the upper end of the pH range, the critical peak pair shifts to impurities A and F and resolution between these analytes decreases, indicating that pH 4.5 would be optimal. To offer a better insight into peak movement as a function of pH value, a separate multimedia file is also provided (**Video 1**).

An increase in temperature decreases retention times for all impurities but has a significant effect only on the separation of impurities C and A, where elevated temperatures are needed for higher resolution values. The gradient time is ideal at around 10 ± 2 min, starting from 10 % ACN and albendazole elutes at 45 % ACN. The accuracy of the predicted retention times and $R_{s,\,crit.}$ values was tested at seven different working points (the obtained data is presented in Supplementary Table 2). A plot of experimentally obtained retention times as a function of predicted values for all analytes under all tested conditions is presented in Fig. 5. As it can be observed, good correlation was obtained between modelled and experimental retention times, moreover, $R_{s,\,crit.}$ values were also in agreement (Supplementary Table 2). Average retention time prediction error was 3.07 %, high values being observed for the first eluting peak, impurity D. When taking into consideration only the most critical analytes, which elute close to each other (impurities E, B, C, A and F), the average retention time prediction error drops to 0.15 %.

Table 2
Experimental and predicted retention times and $R_{s,\,crit.}$ values of the final working point.

Conditions	pH = 4.5 tG = 9 min. (10–45 % ACN) T = 35 °C	
R_t (min.)	Drylab	Exp.
imp. D	1.41	1.56
imp. E	2.52	2.58
imp. B	3.17	3.10
imp. C	3.96	3.88
imp. A	4.45	4.29
imp. F	4.75	4.64
Albendazole	7.57	7.27
R^2	0.9995	
Average retention time error	−0.15%	
$R_{s,\,crit.(imp.\ A–F)}$	3.22	3.40

3.4. In silico *robustness testing and selection of the final method conditions*

Selection of the final method conditions and the software aided *in silico* robustness testing were realized based on the *tG-T-pH* model. Apart from the chromatographic conditions, difference in instrumental variables were also simulated during the testing.

The final method setpoint was selected from the middle part of the 3D region, where $R_{s,\,crit.} \geq 1.5$ was met, as this assured the highest flexibility and appropriate ruggedness of the method, with the following conditions: *pH* = 4.5, *tG* = 9 min. (start[ACN] = 10 %, end[ACN] = 45 %), flow rate = 0.5 mL/min., *T* = 35 °C (average error of retention time prediction = 0.37 %, predicted and experimentally obtained retention times and $R_{s,\,crit.}$ values are presented in Table 2).

Robustness of the method was tested in a multivariate approach, by assessing the effect of seven different parameters at three levels (pH of the sodium acetate buffer 4.5 ± 0.3, flowrate 0.5 ± 0.2 mL/min., column temperature 35 ± 15 °C, gradient time 9 ± 0.5 min, gradient starting ACN 10 ± 2 %, gradient ending ACN 45 ± 2% and dwell volume of the system 0.45 ± 0.1 mL), upon chromatographic method performance. In all the 2187 possible combinations (7 factors at 3 levels) the $R_{s.crit.} \geq 1.5$ criteria is fulfilled with less than 10 min analysis time, indicating a 100 % success rate. The predicted chromatograms for each condition can be visualized and the histogram of the distribution of $R_{s,\,crit.}$ values is also displayed (Fig. 6A). As it can be observed, the highest, simulated $R_{s,\,crit.}$ = 4.07 (Fig. 6B),

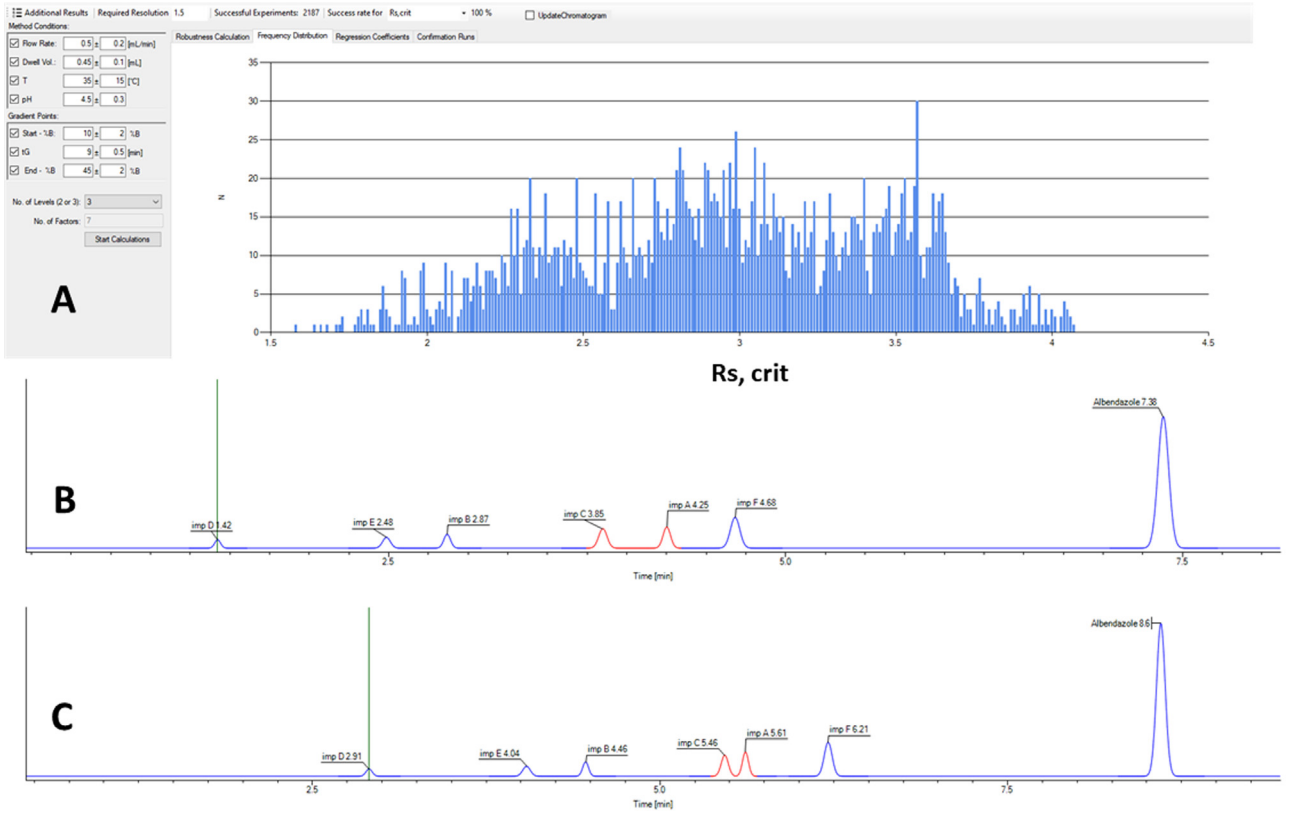

Fig. 6. *In silico* robustness testing of the selected working point **A)** 7 variables (*pH*, *T*, *tC*, flowrate, startB%, tG, endB% and dwell volume) were varied between their individual tolerance limits in $7^3 = 2187$ experiments and the distribution of the predicted $R_{s,\,crit.}$ values are displayed; **B:** the virtual chromatogram in the best-case scenario with $R_{s,\,crit.} = 4.07$ **C:** the virtual chromatogram in the worst-case scenario with $R_{s,\,crit.} = 1.58$.

while even in the worst case-scenario $R_{s,\,crit.} = 1.58$ is obtained (Fig. 6C).

The impact of the individual method parameters and their cross-effect on $R_{s,\,crit.}$ is also visualized on Fig. 7. Based on the obtained results, the combined effects of pH-flow rate and temperature-flow rate present highest impact on $R_{s,\,crit.}$. The effect of dwell volume is important from the point of view of the method transfer between chromatographic systems. The UHPLC system employed in this study was equipped with a quaternary pump with a measured dwell volume of 0.45 mL, but the method also meets the $R_{s,\,crit.} \geq 1.5$ criteria on a chromatographic system equipped with binary pump (lower dwell volume, 0.1−0.2 ml), however in this case the tolerance limits must be defined more rigorously (pH 4.5 ± 0.2, flowrate 0.5 ± 0.1 mL/min., column temperature 35 ± 10 °C).

This systematic investigation without performing the time-consuming, actual experimental runs accelerates method development and enables to select specific working point for variable analytical environment, easing the lifecycle management of the method and the transfer between apparatus and laboratories.

The tolerance limits for routine application were selected based on the robustness study. In this case the parameters can vary within large limits (pH 4.5 ± 0.3, flowrate 0.5 ± 0.2 mL/min., column temperature 35 ± 15 °C, gradient time 9 ± 0.5 min, gradient starting ACN 10 ± 2 %, gradient ending ACN 45 ± 2% and dwell volume 0.45 ± 0.1 mL), therefore the possibility of the Out-of-specification results caused by an underperforming method can be minimized by using the recommended setpoint. A control strategy was also implemented to ensure that the developed UHPLC method is performing as intended. Based on the initial experiments, and *in silico* robustness data a system suitability test (SST) was established, monitoring the resolution of the critical peak pair ($R_{s,\,crit.} \geq 1.5$) between impurities A and F, which has the lowest resolution of all impurity peak pairs.

3.5. Method validation

The short analysis time (< 10 min) of the developed method could render it suitable for not only the quantification of the related substances of albendazole, but the assay of the anthelmintic drug also. Thus, method validation was performed for both impurity testing and assay, according to current ICH guidelines Q2 (R1) by the evaluation of selectivity, sensitivity (only for impurity testing), linearity, accuracy, and precision. The only differences between the assay and impurity method consisted in the final concentration of albendazole (500 μg/mL for the impurity method and 200 μg/mL for the assay)

Based on the information provided by the leaflet of the tablets an artificial placebo powder was reconstituted and a placebo solution was prepared in the same manner as the sample solution. This was done, to identify chromatographic peaks arising from auxiliary substances. The chromatogram of the placebo solution was free from any disturbing effects and unspecified peaks, proving the selectivity of the method. Sensitivity of the impurity method was determined by calculating the limit of detection (LOD) and the limit of quantification (LOQ) values. These were determined based on signal-to-noise ratios of approximately 3:1 and 10:1 for the LOD and LOQ, respectively. The linearity of the impurity method was evaluated at 12 concentration levels, using an artificial mixture of all impurities and albendazole. In the case of the assay method for albendazole quantification, 10 levels were tested. Calibration curves were represented by plotting peak areas against corresponding concentrations (expressed in μg/mL). The correla-

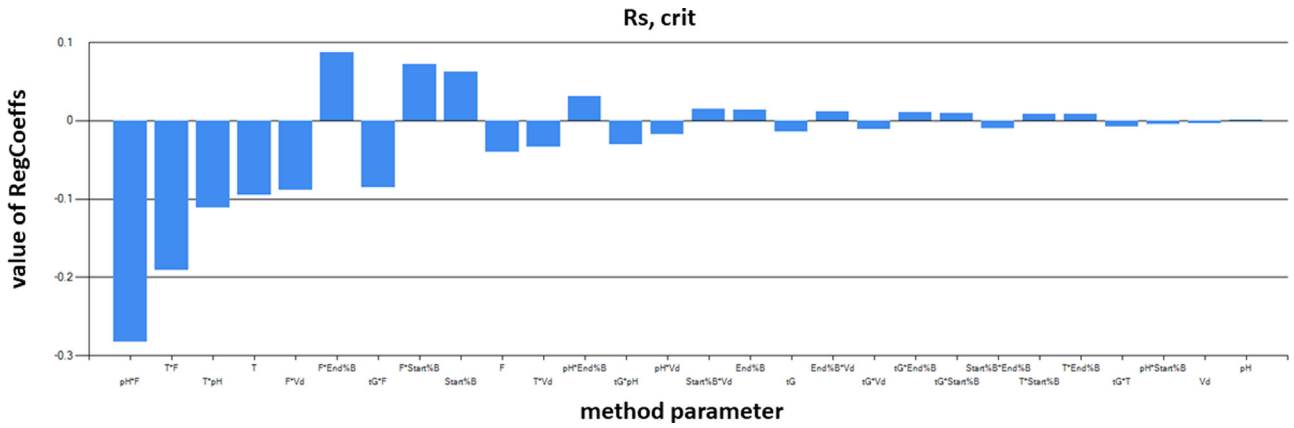

Fig. 7. Relative effects of the chromatographic parameters and their interactions on $R_{s,\,crit.}$

Table 3
Summary of results obtained during method validation.

			Impurity method							Assay
Parameter	Anova F	Level	imp-D	imp-E	imp-B	imp-C	imp-A	imp-F	Albendazole	Albendazole
Range (µg/mL)			0,1–10	0,1–10	0,1–10	0,1–10	0,1–10	0,1–10	0,1–10	100–500
Range (%)			0.02–2	0.02–2	0.02–2	0.02–2	0.02–2	0.02–2	0.02–2	50–250
R^2			0,9999	0,9999	0,9999	0,9999	0,9999	0,9999	0,9996	0,9991
LOD (µg/mL)			0,05	0,03	0,03	0,03	0,03	0,03	0,05	–
LOQ (µg/mL)			0,1	0,1	0,1	0,1	0,1	0,1	0,1	–
RRF			0,65	0,59	0,64	0,63	0,78	0,96	1,00	–
Accuracy (%)		**I***	103,44 ± 1,00	96,92 ± 1,31	100,74 ± 1,37	103,63 ± 0,52	95,91 ± 1,55	100,14 ± 0,90	–	102,49 ± 1,86
		II*	99,87 ± 1,83	98,45 ± 1,61	99,31 ± 2,31	102,52 ± 1,07	97,05 ± 1,00	102,42 ± 0,72	–	102,63 ± 1,30
		III*	100,93 ± 0,51	100,00 ± 0,82	99,34 ± 0,40	101,23 ± 1,00	96,27 ± 0,42	104,16 ± 0,58	–	102,41 ± 1,29
		IV*	99,75 ± 0,56	100,79 ± 0,72	98,37 ± 0,35	100,72 ± 0,94	96,05 ± 0,41	102,33 ± 0,93	–	–
System precision (RSD)			0,52	0,60	1,12	0,60	0,34	0,72	–	0,11
Method Precision (RSD)			1,37	1,75	1,95	1,66	1,60	1,95	–	1,74
Intermediate precision	**$F_{crit.}$**	4,964								
Between days	**F_{calc}**		0,073	1,522	0,001	0,011	0,347	0,044	–	0,614

*impurity method level I: 0.5 µg/mL (0.5 %), level II: 1.0 µg/mL (0.1 %); level III: 3.0 µg/mL (0.6 %) and level IV: 7.5 µg/mL (1.5 %).
*assay method level I: 180 µg/mL (80 %); level II: 200 µg/mL (100 %) and level III: 220 µg/mL (120 %).
F_{calc} – calculated F value; $F_{crit.}$ – critical F value.

tion coefficient was determined by linear least squares regression analysis and its higher than 0.9991 in all cases. For all substances 95 % confidence intervals of the y-intercepts included zero and random distribution of the residuals was observed. The relative response factor (RRF) of the individual impurities were determined as the ratios of the slopes of calibration curves for each impurity in respect to the slope of calibration curve for albendazole.

The accuracy of the impurity method was tested on four different levels, each level with 3 samples, covering the linearity range. The accuracy samples were prepared by adding known amounts of the impurities to the albendazole placebo and the results were evaluated based on the recovery % values. For the assay method the accuracy was evaluated at three levels, adding known amount of albendazole reference substance to the placebo. The accuracy for the impurity method ranged from 95.91 ± 1.55–104.16 ± 0.58 and for the assay from 102.41 ± 1.29–102.63 ± 1.30.

The precision of the system was evaluated by 7 consecutive injections of the same impurity sample (real sample spiked with all impurities) for the impurity method and by 7 repeated injection of the same assay sample (impurity sample diluted 2.5-fold). The precision of both methods was tested by the repetition of the method with 6 real samples. The intermediate precision (between days) was tested by analyzing 6 samples prepared by the same analyst on two consecutive days. The method and system precision were interpreted based on the relative standard deviation (RSD %) values. Regarding method precision the RSD% values ranged from 1.37 % to 1.95 %. For the system precision the RSD% values were between 0.52 % and 1.12 %. The similarity between days was tested using Anova Single Factor analysis and in all cases the $F_{cal.} < F_{crit.}$ criteria were fulfilled. The validation results are summarized in Table 3.

Based on the obtained results the optimized method proved to be selective, sensitive, linear, accurate and precise for the impurity testing and for the assay of albendazole.

3.6. Method applicability on real samples

Applicability of the validated method was tested on real samples in the form of commercially available tablets with a nominal content of 200 mg albendazole. The representative chromatograms for the impurity sample solution with and without spiking with all impurities are shown in Fig. 8A and B, respectively.

Among the monitored related substances, only impurities E, B and F were present in quantifiable amounts in the analyzed samples, the other impurities were below the LOD and LOQ limits. In addition to the known related substances, four minor unidentified peaks could be attributed to unidentified impurities. The total impurity content of the commercial tablets was 0.78 ± 0.01 % (n = 3), which is well below the maximum admitted level (1.30 %) of the Ph. Eur. 10th. The obtained results (mean % ± SD, n = 3) and the relative retention time of the impurities are presented in Table 4.

Albendazole content of the tablets was 192.18 ± 3.65 mg (96.09 ± 1.83 %, n = 6). Based on the results the method is adequate for the analysis of real tablet samples and the analyzed commercially avail-

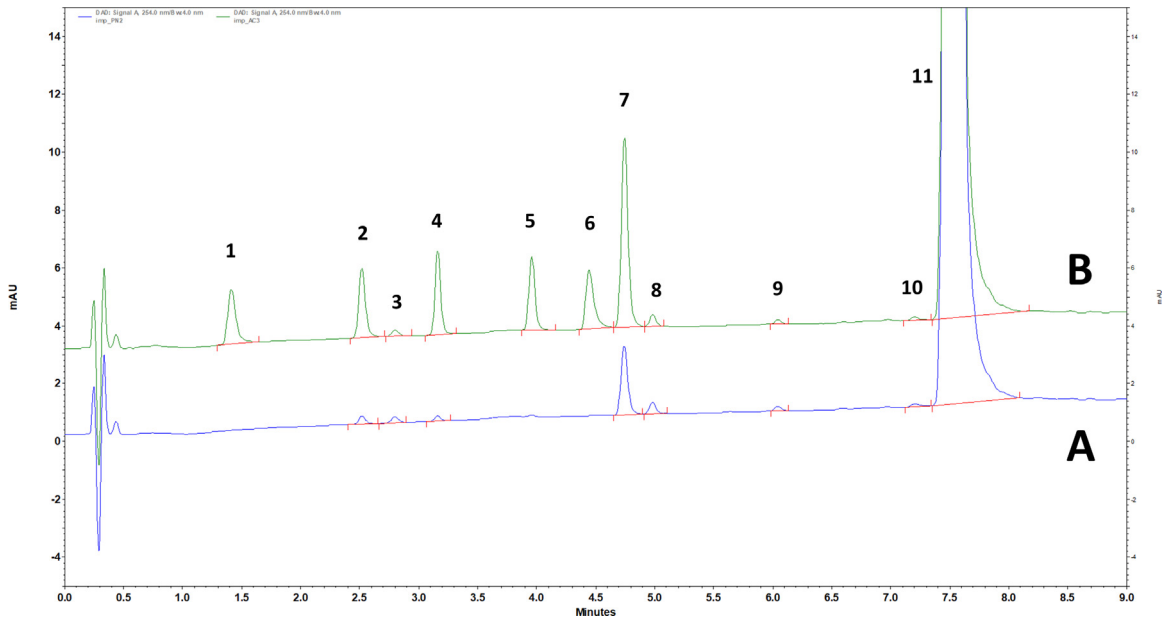

Fig. 8. Chromatograms obtained during method application on a commercial tablet formulation **(A)** Sample solution, **(B)** Sample solution spiked with all impurities (1: impurity D, 2: impurity E, 3: unknown impurity-1, 4: impurity B, 5: impurity C, 6: impurity A, 7: impurity F, 8: unknown impurity-2, 9: unknown impurity-3, 10: unknown-impurity-4, 11: albendazole).

Table 4
Results obtained (mean % ± SD, n = 3) during method application on commercial samples.

Impurity	RRT	Results (%)
impurity D	0.19	< LOD
impurity E	0.33	0.090 ± 0.001
impurity B	0.42	0.048 ± 0.003
impurity C	0.52	< LOD
impurity A	0.59	< LOD
impurity F	0.63	0.479 ± 0.002
unknown impurity-1	0.37	0.042 ± 0.001
unknown impurity-2	0.66	0.072 ± 0.004
unknown impurity-3	0.80	0.026 ± 0.002
unknown impurity-4	0.95	0.025 ± 0.001
Total impurity	–	**0.78 ± 0.01**

able tablets meet the requirements of the Ph. Eur. 10th, regarding to the impurity and active substance content.

4. Conclusions

The Drylab-aided, computerized method development proved to be a fast and reliable way of replacing an underperforming European Pharmacopoeia method with a newly developed, robust, and fast, UHPLC-based technique. A fast preliminary screening of three chromatographic columns was performed using a simple two-dimensional experimental design with only four corner runs. Based and on the obtained models, the most suitable column was selected, and the method further optimized, based on two, tridimensional models. Verification of the experimental models was performed in all cases, by experimental replication of selected working points to prove the validity of the constructed models. Based on the obtained 3D models, optimal regions were visualized, where the baseline resolution criterion was met and an optimal working point was selected from the center of this region, to allow maximum flexibility. *In silico* robustness testing was performed and indicated the appropriateness of the chosen working point. The optimized method was subsequently validated and successfully applied for the impurity testing and the assay of albendazole from commercially available tablet formulation. The results confirm the chromatographic modelling software can not only speed up the analytical method development process, but also improve the reliability of the developed method.

Author statement

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2021.114203.

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