Development of a fast RP-HPLC method for the separation of nifurtimox and its forced degradation products through a design of experiment approach

Giselle Bedogni, Federico Azcárate, Katia Seremeta, Nora Okulik, Claudio Salomon, Carlos Boschetti

PII: S0167-7322(24)00349-0
DOI: https://doi.org/10.1016/j.molliq.2024.124293
Reference: MOLLIQ 124293

To appear in: *Journal of Molecular Liquids*

Received Date: 3 December 2023
Revised Date: 16 February 2024
Accepted Date: 17 February 2024

Please cite this article as: G. Bedogni, F. Azcárate, K. Seremeta, N. Okulik, C. Salomon, C. Boschetti, Development of a fast RP-HPLC method for the separation of nifurtimox and its forced degradation products through a design of experiment approach, *Journal of Molecular Liquids* (2024), doi: https://doi.org/10.1016/j.molliq.2024.124293

Development of a fast RP-HPLC method for the separation of nifurtimox and its forced degradation products through a design of experiment approach

Giselle Bedogni[1], Federico Azcárate[2,3], Katia Seremeta[4], Nora Okulik[4], Claudio Salomon[1,3*], Carlos Boschetti[2,3*]

[1] Instituto de Química Rosario, Consejo Nacional de Investigaciones Científicas y Técnicas (IQUIR-CONICET). Suipacha 570, Rosario 2000, Argentina; bedogni@iquir-conicet.gov.ar (G.B.); csalomon@fbioyf.unr.edu.ar (C.S.)

[2] Instituto de Procesos Biotecnológicos y Químicos, Consejo Nacional de Investigaciones Científicas y Técnicas (IPROBYQ-CONICET); azcarate@iprobyq-conicet.gob.ar (F.A.); cboschet@fbioyf.unr.edu.ar (C.B.)

[3] Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario. Suipacha 570, Rosario 2000, Argentina

[4] Instituto de Investigaciones en Procesos Tecnológicos Avanzados, Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional del Chaco Austral (INIPTA-CONICET-UNCAUS). Cte. Fernández 755, Presidencia Roque Sáenz Peña 3700, Argentina; kseremeta@uncaus.edu.ar (K.S.); nora@uncaus.edu.ar (N.O.)

*Corresponding authors e-mail address: csalomon@fbioyf.unr.edu.ar (Claudio Salomón - Suipacha 570, Rosario 2000, Argentina); cboschet@fbioyf.unr.edu.ar (Carlos Boschetti - Suipacha 570, Rosario 2000, Argentina)

Keywords

Design of Experiments, Method development, Nifurtimox, HPLC

List of abbreviations

NFX: nifurtimox; RP-HPLC: reverse-phase high-performance liquid chromatography; ICH: International Conference on Harmonization; DoE: Design of Experiments; ANOVA: Analysis of variance; Res: Resolution; T_{ret}: Retention Time; T_f: Tailing Factor; LoD: Limit of Detection; LoQ: Limit of Quantification

Abstract

The study depicts the successful development and validation of a simple and fast reverse-phase high-performance liquid chromatography (RP-HPLC) method for the separation of nifurtimox (NFX) and its degradation products. NFX was exposed to different degradation conditions and a RP-HPLC method reported in the literature was used to evaluate different peaks that could interfere with the one from the pure NFX. NaOH generated a degradation product, with a peak that tends to overlap with NFX. Therefore, the NFX exposed to this condition was used during screening and optimization of the RP-HPLC method using a Design of Experiment (DoE) approach. A factorial design was applied to screen different factors, followed by a central composite design for process optimization with those factors that significantly affected the evaluated responses during the screening step. The optimized method was further validated according to International Conference of Harmonization guidelines. Methanol proportion and gradient time to reach it, alongside with mobile phase flow rate, were the factors that had a statistically significant effect on resolution, retention time, and tailing factor in the chromatographic peak. The optimized chromatographic parameters were: 41.6% of methanol, a gradient time of 2 min, and a flow rate of 0.62 mL/min. Under these chromatographic conditions, the retention time of NFX was 5.3 min, while for the nearest degradation products was 4.5 min and 6.3 min. The resolution of the NFX peak was 5.14 and the tailing was 1.13. The DoE approach allowed the development of an optimized RP-HPLC method capable of successfully separating NFX and its forced degradation products.

1. Introduction

Nifurtimox (NFX, IUPAC name: (E)-N-(3-methyl-1,1-dioxo-1,4-thiazinan-4-yl)-1-(5-nitrofuran-2-yl) methanimine) is a nitrofuran derivative drug. The chemical structure of NFX (Fig. 1) shows that degradation is a very feasible problem since a heteroaromatic ring rearrangement is possible or even the cleavage and oxidation/reductions in double bonds through chemical breakdown [1,2]. NFX also contains a nitrofuran group, enhancing the need for control over genotoxic degradation products [3].

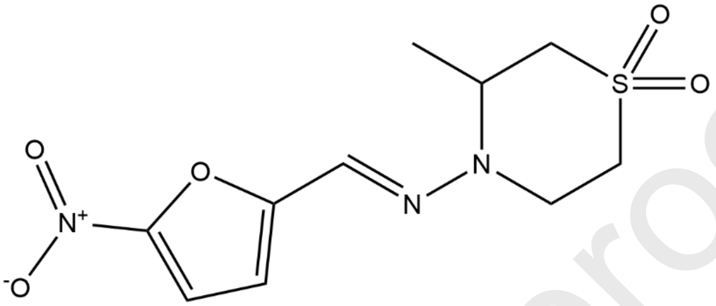


Fig. 1. Chemical structure of nifurtimox. IUPAC name: (E)-N-(3-methyl-1,1-dioxo-1,4-thiazinan-4-yl)-1-(5-nitrofuran-2-yl) methanimine.

The International Conference on Harmonization (ICH) guidelines describe degradation products as "impurities resulting from a chemical change in the drug substance brought about during manufacture and/or storage of the new drug product by the effect of, for example, light, temperature, pH, water, or by reaction with an excipient and/or the immediate container closure system" [4]. To date, no study has reported the degradation or stability of NFX under stress conditions.

One of the most common and important tools to analyze drug identity, drug content, and impurities in the pharmaceutical field is the High-Performance Liquid Chromatography (HPLC) [5]. The lack of studies regarding NFX impurities and forced degradation products is a problematic gap in knowledge related to quality control. It is important to note that NFX is used for the treatment of Chagas disease, a neglected tropical disease. Chagas disease is mainly distributed across Central and South America, where climate characteristics present a wide range of temperature and humidity, which could exacerbate stability-related problems for NFX [6,7].

Design of Experiments (DoE) is a systematic approach to optimize a process or method, based on predefining goals and putting emphasis on product and process understanding, while performing a limited number of experiments [8]. Designs are organized in such a way that different factors are being simultaneously evaluated at different levels, on different combinations [9–11]. This allows us to evaluate not only critical factors but also how the interaction between those factors and their levels influences the evaluated responses. Collected data is submitted to different mathematical models to evaluate the statistical impact of different factors and make further predictions about optimized combinations. DoE has been successfully applied for HPLC method development, whether for drug quantification or for drug separation from degradation products and simultaneous quantification as reported by several authors, demonstrating the usefulness of this approach [12–17].

In this study, NFX was exposed to several conditions to force its degradation. When exposed to NaOH, hydrolysis produces a degradation product, whose elution time is critically close to pure

NFX, which can affect drug quantification. Therefore, a DoE approach was applied for the development of an RP-HPLC method, capable of separating the NFX peak from the nearest degradation product peak. First, a factorial design was used to test different factors that tend to affect the elution times of pure drugs and their degraded products or related impurities. With the information collected from the performed screening, significant factors were identified. Next, an optimization step was carried out using those significant factors in a central composite design, and appropriate response surface models were obtained. Afterward, the optimized RP-HPLC method was validated according to the requirements established by the ICH.

2. Materials and methods

2.1. Materials

NFX was kindly donated by Gador (~99.998 % purity, Gador, lot 160209, Buenos Aires, Argentina). Hydrochloric acid (37% w/w, lot 18.0230202.5000), sodium hydroxide (pellets, lot DCBB0925), and hydrogen peroxide (30% w/w, lot 60327) were purchased from Chem-Lab (Zedelgem, Belgium), Sigma Aldrich (Darmstadt, Germany) and Cicarelli (Santa Fe, Argentina) respectively. All chemical reagents were HPLC grade.

2.2. Forced degradation

NFX was exposed to hydrolytic and oxidative conditions to obtain different degradation products. Different sets of suspension of NFX (1 mg/mL) were prepared with HCl (0.1 M and 1 M), NaOH (0.1 M and 1 M) and H_2O_2 (1.5 %v/v and 3 %v/v), and stored at 25 °C or 60 °C for 48 h. Subsequently, samples were filtered, and neutralized and chromatographic runs were performed under chromatographic conditions previously reported for NFX [18–21]. An isocratic mode, with a mobile phase consisting of 50% methanol (MeOH) and 50% acetic acid 0.2% in water, at a flow rate of 0.5 mL/min was used. The column used was a reverse phase BDS Hypersil C18 (Thermo Scientific™ Inc, United States) of 100 x 4.6 mm and 2.4 µm particle size. The injection volume was 10 µL, the run duration was 10 min and the column temperature was set at 25 °C. HPLC instrument is further detailed in section 2.4.1.

2.3. Design of experiment

2.3.1. Screening

A fractional factorial design with resolution V was applied to identify significant factors for the chromatographic separation. Five independent factors were evaluated: concentration of organic modifier in the mobile phase (MeOH, %v/v), gradient time (Time, min), pH of aqueous mobile phase, column temperature (Temperature, °C), and flow rate (mL/min). Mentioned factors were tested at two levels, and their effect on responses was studied with Minitab statistical software (Minitab® 17.1.0, Pennsylvania, USA), which was also used to generate a randomized design, with 16 runs (Table 1). Responses evaluated were categorized into main responses: resolution (Res), retention time (T_{ret}), and tailing factor (T_f), and secondary responses: peak area and peak height. Each run was performed in triplicate, and the responses were statistically analyzed by ANOVA. Differences were considered statistically significant for p-values < 0.05.

Run	**Independent factors**	**Responses**

	Time (min)	MeOH (%)	pH	Temperature (°C)	Flow rate (mL/min)	Res	T_{ret} (min)	T_f	Peak area	Peak height
1	0	40	3	40	0.4	0.000	8.431	1.093	1.719	9.699
2	0	50	6	40	0.4	1.970	5.226	1.187	1.857	16.600
3	3	40	6	40	0.4	0.000	7.221	1.143	1.820	16.136
4	3	50	6	40	0.6	2.020	3.501	1.183	1.231	16.928
5	3	50	3	40	0.4	2.130	5.195	1.177	1.702	15.266
6	3	50	6	30	0.4	1.793	5.754	1.223	2.207	17.744
7	3	40	3	30	0.4	-2.890	7.793	1.157	1.720	14.842
8	0	40	6	30	0.4	0.000	9.805	0.990	1.632	8.848
9	0	50	6	30	0.6	1.763	3.827	1.183	1.432	17.470
10	3	50	3	30	0.6	0.970	3.764	1.200	1.133	14.235
11	0	40	6	40	0.6	0.000	5.635	1.110	1.199	10.641
12	0	50	3	40	0.6	0.000	3.474	1.193	1.121	15.368
13	3	40	3	40	0.6	-2.143	5.050	1.113	1.207	15.319
14	0	40	3	30	0.6	0.000	6.502	1.095	1.617	11.775
15	0	50	3	30	0.4	1.545	5.659	1.170	1.714	14.361
16	3	40	6	30	0.6	-2.235	5.500	1.137	1.210	15.047

16	3	40	6	30	0.6	-2.670	5.560	1.130	1.438	17.459

Table 1. Experimental condition of the factorial design for screening of critical factors. Non-coded values are presented and responses are expressed as the mean (n=3).

2.3.2. Optimization

Based on the results of the screening, a central composite design was used for the optimization of main responses. Thus, the significant independent factors selected in the screening step were next evaluated at five levels. For gradient time, the superior and inferior values were set as 0 min and 5 min, for MeOH concentration the limits were 40% and 60%, and for flow rate, they were 0.3 mL and 0.7 mL/min, respectively. Table 2 summarizes the randomized design given by Minitab statistical software (Minitab® 17.1.0, Pennsylvania, USA). Each run was performed in triplicate and mathematical models for each response were evaluated by ANOVA (differences were considered statistically significant for p-values < 0.05).

Run	**Independent factors**			**Responses**				
	Time (min)	**MeOH** (%)	**Flow rate** (mL/min)	**Res**	**T_{ret}** (min)	**T_f**	**Peak area**	**Peak height**
1	2.50	50.00	0.50	1.260	4.520	1.195	1.500	12.408
2	4.00	56.00	0.62	3.157	2.834	1.257	1.282	16.381
3	2.50	50.00	0.50	1.270	4.510	1.200	1.895	13.050
4	2.50	60.00	0.50	1.730	3.113	1.290	1.488	19.974
5	1.00	44.00	0.62	1.693	4.544	1.163	1.288	13.870
6	1.00	56.00	0.62	5.05	2.82	1.24	1.297	17.247

				0	6	0		
7	4.00	44.00	0.62	2.360	4.779	1.153	1.288	12.156
8	2.50	50.00	0.30	1.660	7.257	1.200	2.471	12.802
9	4.00	56.00	0.38	3.087	4.564	1.230	2.145	16.913
10	0.00	50.00	0.50	1.927	4.143	1.217	1.595	17.477
11	2.50	50.00	0.50	1.340	4.508	1.180	1.582	13.116
12	2.50	40.00	0.50	5.860	6.740	1.140	1.431	10.126
13	2.50	50.00	0.50	1.260	4.512	1.200	1.574	13.032
14	1.00	56.00	0.38	3.063	4.543	1.240	2.135	17.973
15	2.50	50.00	0.70	1.130	3.287	1.170	1.071	11.475
16	4.00	44.05	0.38	1.877	7.522	1.153	2.119	12.578
17	1.00	44.00	0.38	1.200	7.181	1.160	2.109	13.662
18	2.50	50.00	0.50	1.32	4.50	1.19	1.600	13.152

				0	8	0		
19	5.00	50.00	0.50	1.250	4.583	1.210	1.571	11.641
20	2.50	50.00	0.50	1.290	4.523	1.185	1.630	13.358

Table 2. Central composite design for the optimization of RP-HPLC method. Responses are expressed as the mean value for a n=3.

2.4. Method validation

The optimized method was validated based on ICH requirements from the Guideline Q2(R1) [20].

Linearity: A standard calibration curve was prepared with concentrations ranging between 2.50 μg/mL and 20 μg/mL. Three replicate injections of each concentration were analyzed and linear regression and correlation coefficient were obtained from the graph of peak area versus concentration.

Limit of detection and limit of quantitation: the sigma method was used for the limit of detection (LoD) and limit of quantification (LoQ) applying Eq. (1) and Eq. (2), where σ is the standard error obtained from the regression analysis and S is the slope of the calibration curve.

$$\mathrm{LoD} = (3.3\sigma)/\mathrm{S} \quad (1)$$

$$\mathrm{LoQ} = (10\sigma)/\mathrm{S} \quad (2)$$

Accuracy: three different standard concentrations were measured in triplicate to test the accuracy of the calibration curve. The concentrations tested were 4, 7 and 14 μg/mL.

Precision: for interday precision, triplicate measurements of low (5 μg/mL), medium (13 μg/mL), and high (17 μg/mL) concentrations of NFX were taken. The mean, SD, and RSD of the peak areas and corresponding concentrations were calculated. The acceptance limit for precision was that the RSD of the drug should not exceed 2%.

2.5. Instrumentation and chromatographic conditions

2.5.1. Instrument

Chromatographic analysis was performed on HPLC equipment (Dionex^{TM} UltiMate^{TM} 3000, Thermo Scientific^{TM} Inc, Massachusetts, United States) with a photodiode array and fluorescence detectors (DAD-3000 and FLD-3100 PMT), a standard quaternary pump (LPG 3400SD) and autosampler column compartment (ACC-3000T). The column used was a reverse phase BDS Hypersil C18 (Thermo ScientificTM Inc, United States), with dimensions of 100 x 4.6 mm and 2.4 μm particle size. The injection volume was 10 μL. Detection was performed at 400 nm. Control was carried out by Chromeleon software (version 7.2 SR4). The mobile phase was a mixture of methanol and a solution of acetic acid 0.2% in water.

For the designed experiments, gradient programs were the following: for those experiments where gradient time was 0, the isocratic mode was used (either 40% or 50% MeOH in aqueous acetic acid), while in those experiments where time was different than 0, the organic modifier was increased from 40% to the required final concentration in the correspondent time (1, 2.5, 4 or 5 min), remaining constant after that. After the optimization step, the resulting chromatographic conditions were: initial 40% MeOH, gradient to 41.6% in 2 min, at a flow rate of 0.62 mL/min.

2.5.2. Working solution

Working solutions of degraded samples were prepared in basic conditions (0.1 NaOH M, kept at 60 °C for 48 h). The samples were subsequently neutralized with HCl 0.1 M and filtered.

3. Results and discussion

3.1. Forced degradation

During forced degradation studies, drugs are exposed to stress conditions under which they can undergo degradation through different pathways, whose products could eventually affect treatment efficacy or generate toxic products [23]. The degradation process can be accelerated after exposure to light, high temperatures, and other stress conditions [24]. As it was stated before, there is a lack of knowledge about the degradation pathways of NFX, nonetheless, forced degradation studies have been previously conducted on other drugs that share the nitrofuran structure with NFX. Biosic *et al* elucidate nitrofurantoin degradation pathway under hydrolytic conditions at different pH values, which resulted in six different degraded products [25]. Nitrofurantoin can also form nitrofuraldehyde after photohydrolysis [26]. Nitrofurazone is another nitrofuran derivative that can form 8 dominating intermediates after the redox process during the generation of degradation products [27].

Here, the stability of NFX under hydrolytic (NaOH and HCl) and oxidative conditions was evaluated. Based on previously reported HPLC methods for NFX, the separation of NFX and degradation products (if generated) was carried out in an isocratic mode, with a mobile phase consisting of 50% MeOH and 50% of a 0.2% solution of acetic acid in water, at a flow rate of 0.5 mL/min. Under these chromatographic conditions, pure NFX dissolved in acetonitrile had a T_{ret} of 4.78 min.

From all the assayed experimental conditions, only the degradation products from basic hydrolysis presented a closely eluting impurity near the NFX peak, as can be seen in Fig. 2. T_{ret} was 2.09, 4.56, and 7.46 min for those products. Also, a broad signal was observed around 3.61 min. It has been reported that furazolidone generated degradation products in NaOH 0.01 N conditions (with a drug recovery of 4.98 %, indicating a vast degradation extension) and the appearance of three additional peaks were observed [28].

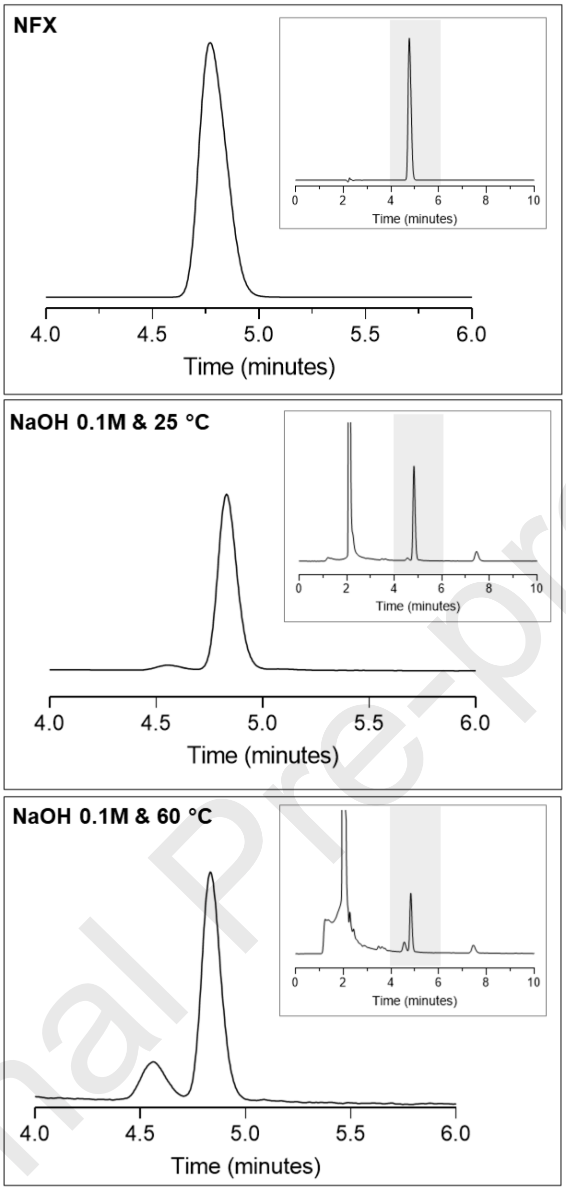


Fig. 2: Chromatograms of NFX without exposure to degradation conditions and NFX after exposure to basic hydrolysis (NaOH 0.1 M) at two temperatures (25 °C and 60°C). Insert shows full chromatograms (10 min run).

After exposure to acidic hydrolysis and to H_2O_2 for 48 h, the generated degradation products had elution times that did not interfere with pure NFX (1.41 min with a small absorbance value for H_2O_2 and a peak with high absorbance at 2.08 min for acidic hydrolysis). Therefore, we used the hydrolyzed products that were generated under basic conditions to develop a new method for NFX separation by applying a DoE approach.

3.2. Design of experiment

3.2.1. Screening step

Fractional factorial designs are a reduced version of full factorial design, where different factors that could potentially affect the responses of interest are evaluated. Use of the fractional factorial allows a reduction in the number of runs and so a more efficient use of resources [29]. A fractional factorial design at two levels was applied to screen five factors and evaluated which had a significant impact on critical parameters for the separation between the NFX peak and the closest peak from basic hydrolysis. According to the half fraction factorial design, $2^{5-1} = 16$ experiments were conducted and the results can be found in Table 1. The resolution value of the chosen design (V) allowed to keep the power of the design and the ability to differentiate between main effects and interactions of factors.

Pareto charts were obtained from ANOVA results. From the Pareto charts in Fig. 3, it can be seen that, by far, the factor that mainly influences most responses is MeOH concentration. Variation in MeOH concentration affects the viscosity and allows different polarities in the mobile phase, thus altering the interaction among the column and NFX and its degradation product, even those with possible high structural similarities. Gradient time and flow rate also showed statistical significance in some of the analyzed responses.

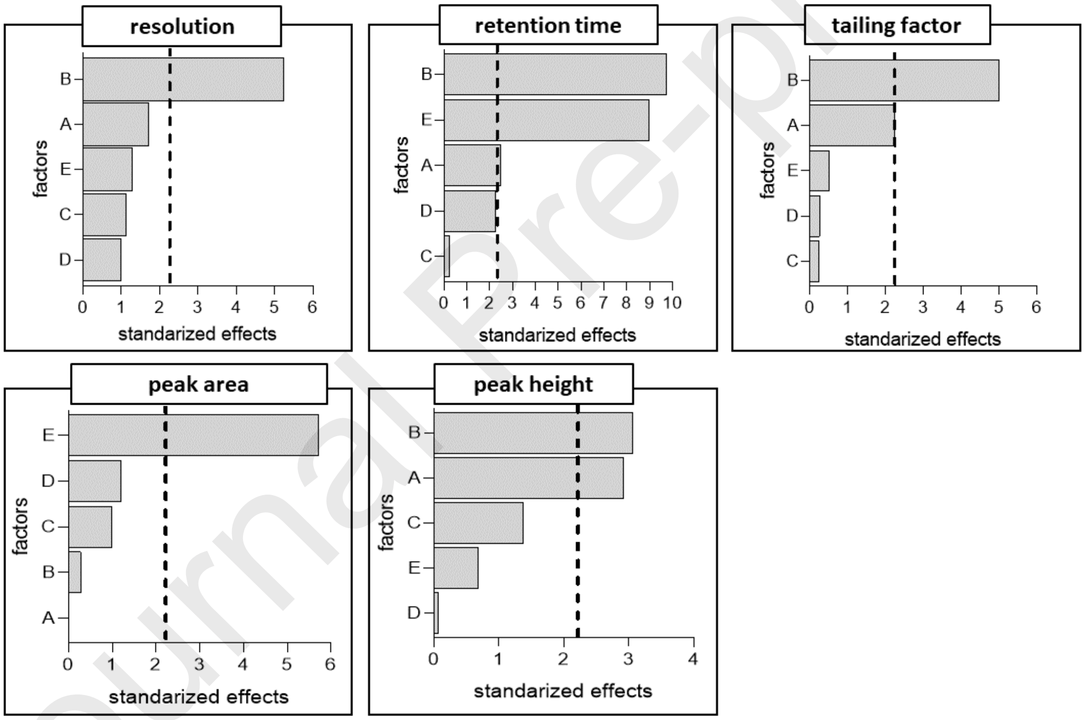


Fig. 3. Pareto charts from the screening experiment, showing the effect of each factor (A: Time, B: MeOH, C: pH, D: Temperature, and E: Flow rate) over different evaluated responses. The reference line for statistical significance is plotted as a dotted line at 2.201.

Collected data was analyzed with an ANOVA test. A linear model was applied to model the responses, showing adequate fit and lack of fit values and a good relationship between the values of the coefficient of determination (R^2) and the adjusted R^2 (R^2ad) (Table 3).

	Factors	**Model**

		Time	**MeOH**	**pH**	**Temperature**	**Flow rate**	**Fit**	**Lack of fit**	**R^2**
Res[a]	p-value	0.112	**<0.0001**	0.24	0.319	0.206	0.003	0.231	R^2: 0.7764
	coded coefficient	-0.275	0.2552	0.198	0.0498	-3.21			R^2ad: 0.6748
T_{ret}[b]	p-value	**0.031**	**<0.0001**	0.872	**0.045**	**<0.0001**	<0.0001	0.06	R^2: 0.9396
	coded coefficient	-0.315	-1.205	0.021	-0.288	-1.131			R^2ad: 0.9121
T_f[c]	p-value	**0.044**	**0.001**	0.796	0.778	0.579	0.008	0.098	R^2: 0.7186
	coded coefficient	0.01977	0.0421	-231	0.00252	0.00498			R^2ad: 0.5906
Peak area	p-value	0.989	0.782	0.327	0.242	**<0.0001**	0.003	0.6	R^2: 0.7656
	coded coefficient	0.0006	0.0126	0.0456	-0.055	-0.2594			R^2ad: 0.6590
Peak height	p-value	**0.014**	**0.011**	0.204	0.965	0.513	0.029	0.581	R^2: 0.6365

coded coefficient	1.422	1.48	0.659	-0.022	0.33		R^2ad: 0.4713

[a] Res: Resolution; [b] T_{ret}: Retention time of NFX, [c] T_f: Tailing factor,

Table 3. ANOVA results from screening experiments. Factors that induced a statistically significant difference in responses can be found in bold format (p-value<0.005).

MeOH concentration had a significant impact on Res ($p < 0.0001$), T_{ret} ($p < 0.0001$), and T_f ($p = 0.001$). Usually, the degraded product tended to elute before NFX, but when MeOH concentration was 50% (and gradient time 3 min), in some chromatographic runs it eluted after NFX, hence the negative values in Res shown in Table 1. Gradient time had a slightly significant influence in T_{ret} ($p = 0.031$) and T_f ($p = 0.044$) whereas temperature only showed borderline significance in T_{ret} ($p = 0.045$). T_{ret} was highly affected by flow rate ($p < 0.0001$), with considerably reduced elution times at the maximum flow rate tested.

Other responses that could be evaluated from chromatograms were peak area and peak height (considered as secondary responses), therefore, they were measured to study pure NFX peak. Peak height was affected by time and MeOH ($p = 0.014$ and $p = 0.011$, respectively), while peak area was highly influenced by flow rate ($p < 0.0001$).

Based on these results, time, MeOH, and flow rate were chosen to carry out the optimization process. The column temperature was set at 40 °C since lower T_{ret} values were obtained with higher temperatures, as indicated by the coefficient value (-0.288). Since the pH of the mobile phase had no statistical influence in any of the evaluated responses, only water was used.

3.2.2. Optimization step

The purpose of this work was to develop a fast RP-HPLC method capable of separating NFX from degradation products that presented similar T_{ret} in a single chromatographic run. Based on the results obtained in the screening step, the following optimization step was performed setting time gradient, MeOH, and flow rate as the independent variables. In order to use a response surface methodology, a central composite design was chosen because it allows the levels for each factor to be enlarged with center and axial points, and its rotatability provides a robust design with an excellent prediction capability [30]

The results of the central composite design are shown in Table 2. ANOVA was applied to evaluate a quadratic model for regression of the response surface. The procedure to analyze each response was first to model the data into a quadratic equation including all terms (quadratic, interactions, and linear terms). If the fitting was not good, outliers were checked and then elimination of non-significant terms was performed.

Res and T_{ret} data were parametrically transformed in order to set a model with good statistical fit. The suitability of Yeo-Johnson parametric transformation has been proved by Atkinson *et al* and it was previously used during the analysis of different DoE, showing that Yeo-Johnson transformation allows precise and accurate regression models [31-34]. Therefore, we applied this transformation to perform a parametric transformation for Res and T_{ret} to obtain a normal distribution and an appropriate model fit (Table 4). Transformed data was analyzed through a normality test to confirm an appropriate transformation was performed. Ryan-Joiner test was applied and there was no evidence that data did not follow a normal distribution ($p>0.05$). For the remaining responses, no transformation was needed and models showed good fit (see Table 4).

	Quadratic model	
	Model p-value	**Lack of fit**
Res$^{(a,b)}$	<0.0001	0.085
$T_{ret}^{(a,b)}$	<0.0001	0.733
T_f	<0.0001	0.244
Peak area	<0.0001	0.192
Peak height	<0.0001	0.604

^{a}: Johnson transformation; ^{b}: outlier elimination

Table 4. ANOVA results from the optimization step through central composite design (p-value<0.005).

Regression equations in non-coded units for optimization are: ***Res_JT*** = 0.03518 *MeOH*^{2} + 0.269 *Time* - 3.417 *MeOH* + 2.847 *Flow rate* +80.80; ***T_{ret}_JT*** = 0.0745 Time^2 +0.002436 *MeOH*^{2} + *6.69 Flow rate*^{2} + 0.00371 *Time*MeOH* + 0.543 *Time*Flow rate* - 0.3559 *MeOH*Flow rate* - 0.747 *Time* - 0.1705 *MeOH* + 5.29 *Flow rate* + 7.06; ***T_f*** = +0.00309 *Time*^{2} + 0.000210 *MeOH*^{2} -0.01648 *Time* -0.01371 *MeOH* -0.0126 *Flow rate* + 1.379; ***Peak Area*** = 0.01030 *Time*^{2} - 0.000590 *MeOH*^{2} + 6.31 *Flow rate*^{2} - 0.0532 *Time* + 0.0608 *MeOH* - 9.83 *Flow rate* + 3.41; ***Peak height*** = 0.5414 *Time*^{2} +0.03459 *MeOH*^{2} - 22.79 *Flow rate*^{2} - 3.113 *Time* - 3.115 *MeOH* + 20.51 *Flow rate* + 82.15.

Contour plots for main modeled responses are shown in Fig. 4. In a preliminary view of such plots it can be seen that a high flow will favor minimizing T_{ret} and T_f while it maximizes Res. The effect of the remaining factors is not evident. Low MeOH could produce minimum values of T_f and high Res, but T_{ret} will be enlarged. Time presents opposite effects when trying to maximize Res and reduce T_{ret}. Therefore, to get a single set of conditions for the three responses to be favored, the use of the desirability function is needed.

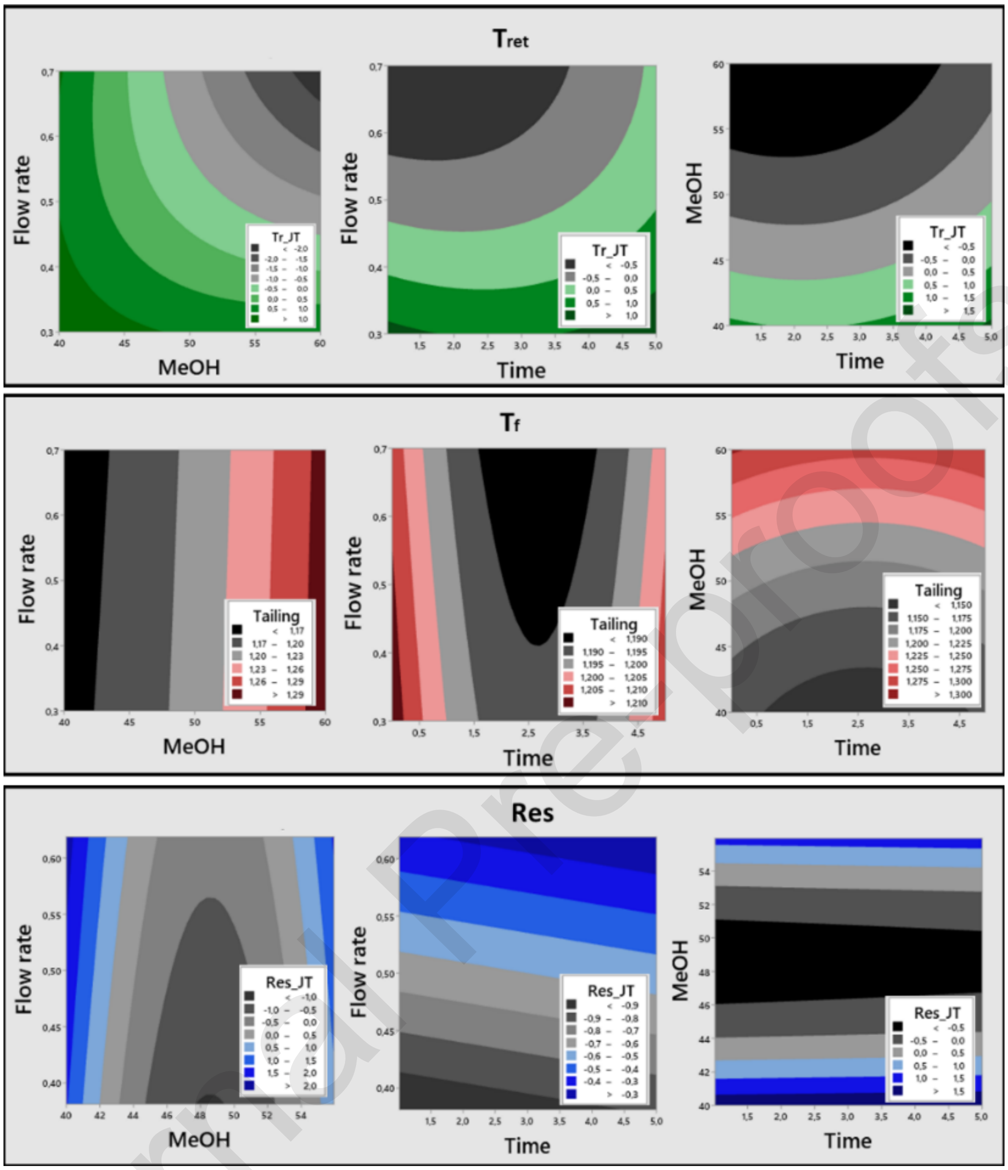


Fig. 4. Contour plot of main responses.

Since the main goal of this study was to obtain the conditions in which higher separation between the NFX peak and nearest degradation product peak was achieved, the optimization criteria for the three factors and all responses were set to maximize Res (importance of 3), minimize T_f and T_{ret} (with an importance of 1). Optimized conditions, model-predicted values, and experimental values of Res, $T_{f,}$ and T_{ret} are presented in Table 5. Under these chromatographic conditions, four chromatographic peaks from degradation products are present at the following T_{ret}: 4.17 min, 4.49 min, 6.29 min, and 6.38 min. The predictability of the model was analyzed in terms of the percentage of prediction error (PE) according to Eq. 3, where Exp is the experimental value obtained for each response and Pred is the predicted value calculated by the mathematical model used [35].

$$PE = 100 * (Pred - Exp)/Pred \quad (3)$$

Values	**Time** (min)	**MeOH** (%)	**Flow rate** (mL/min)	**T_f**	**T_{ret}** (min)	**Res**	**Desirability**
Predicted	1.9799	41.6107	0.6189	1.1376	5.9071*	4.2788*	0.9376
Experimental	2	41.6	0.62	1.13	5.313	5.14	
PE				**0.67%**	**10.06%**	**20.13%**	

Table 5. Predicted and experimental values from optimized method (*: predicted values calculated from linear regression of transformed data from central composite design).

Experimental values were obtained from new runs in triplicate, setting the required values for the studied factors, *i.e.*, Time, MeOH, and Flow rate. The average measured results are in agreement with the predicted ones, as can be seen by the PE. With the initially tested chromatographic method, based on literature references, T_{ret} for NFX was 4.78 min and the Res with the closest degradation product peak was 1.41. With the optimized method developed here, an appropriate T_f was achieved in a reasonably short T_{ret} of 5.31 min, whereas the Res value was increased up to 5.14, indicating that the method is capable of separating pure NFX from molecules formed during its degradation.

3.3. Method validation

Under the optimized chromatographic conditions, the average T_{ret} for NFX was 5.103 min ± 0.006. The linearity of the method was evaluated in a concentration range between 2.50 and 20.00 µg/mL. From the chromatograms, peak areas were plotted against the concentration values, and regression analysis was performed. As can be seen in Fig. 5, a linear relationship between NFX concentration and area under the peak was obtained, with a correlation coefficient R^2 >0.99.

LoD and LoQ were calculated with the data from the calibration curves, and their values resulted in 1.06 and 3.21 µg/mL, respectively.

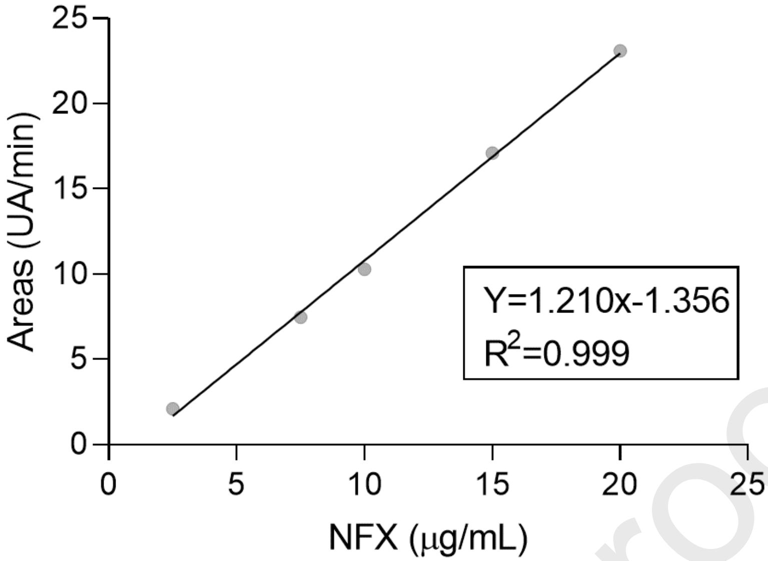


Fig. 5. Calibration curve obtained with average values of peak areas for five different NFX concentrations.

Accuracy across the analytical range showed good recovery percentages for NFX at tested concentration (between 95 to 105 %), while intraday precision had RSD less than 2 % (Table 6).

NFX$_T$ [a] (µg/mL)	**NFX**$_M$ [b] (µg/mL)	**Accuracy** (%)	**Intraday Precision** (RSD%)
5	5.01 ± 0.06	100.12 ± 1.18	1.18
13	12.98 ± 0.05	99.81 ± 0.40	0.40
17	17.25 ± 0.03	101.44 ± 0.19	0.19

[a]NFX$_T$: Theoretical concentration of NFX, [b]NFX$_M$: average intraday concentration measured of NFX

Table 6. Intraday method validation.

4. Conclusion

Force degradation study of NFX was performed and it was found that NFX was susceptible to hydrolyzation under acidic and basic conditions, while it showed higher stability when exposed to H_2O_2. Selecting the conditions for HPLC analysis is a challenging step since it depends on multiple variables. A new RP-HPLC method for the separation of NFX and its degradation products under basic conditions was developed applying a DoE approach. The optimized method allows to separate closely eluting peaks using a mobile phase with 41.6% of MeOH in water, with a gradient time of 2 min and a flow rate of 0.62 mL/min, achieving a Res of 5.14, with T_{ret} of 5.10 min for pure NFX and T_f under 1.13. This optimized method had a linear response to increasing concentration of NFX, with LoD and LoQ of 1.06 and 3.21 μg/mL, respectively. It also showed good accuracy and precision.

Funding

This work was supported by Agencia Santafesina de Ciencia, Tecnología e Innovación (PEICID-2021-084), Universidad Nacional de Rosario (PID 80020180100134UR), CONICET (PIP 3179) and MINCYT (PICT 2019-4180).

Acknowledgment

GB acknowledges CONICET for a doctoral fellowship.

CRediT

Giselle Bedogni: Conceptualization, Formal analysis, Methodology, Investigation, Visualization, Writing - original draft, review & editing. Federico Azcárate: Data curation, Investigation, Methodology. Katia Seremeta: Writing - review & editing. Nora Okulik: Writing - review & editing. Claudio Salomon: Conceptualization, Funding acquisition, Resources, Writing - review & editing. Carlos Boschetti: Conceptualization, Funding acquisition, Resources, Project administration, Writing - review & editing.

References

[1] Gabrič A, Hodnik Ž, Pajk S. Oxidation of Drugs during Drug Product Development: Problems and Solutions. Pharmaceutics, 14(2) (2022), 325. https://doi.org/10.3390/pharmaceutics14020325

[2] Liu DQ, Kord AS. Analytical challenges in stability testing for genotoxic impurities. Trends Analyt. Chem., 49 (2013) 108–117. http://dx.doi.org/10.1016/j.trac.2013.06.004

[3] Müller L, Mauthe RJ, Riley CM, Andino MM, Antonis D De, Beels C, *et al*. A rationale for determining, testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity. Regul. Toxicol. Pharmacol., 44(3) (2006) 198–211. doi:10.1016/j.yrtph.2005.12.001

[4] International Conference on Harmonization. Guideline Q3B(R2). 2006.

[5] Nikolin B, Imamovic B, Medanhodzic-Vuk S, Sober M. High Perfomance Liquid Chromatography In Pharmaceutical Analyses. Bosn. J. Basic. Med. Sci., 4(2) (2004) 5–9.

[6] Browne AJ, Guerra CA, Alves RV, Da Costa VM, Wilson AL, Pigott DM, *et al*. The contemporary distribution of *Trypanosoma cruzi* infection in humans, alternative hosts and vectors. Sci. Data, 4 (2017) 170050. DOI: 10.1038/sdata.2017.50

[7] Jenkins D, Harmon CL, Jia X, Kesselring A, Hatcher D, Grayson K, Ayres, J. Forced degradation studies of medroxyprogesterone acetate injectable suspensions (150 mg/ml) with implementation of HPLC, mass spectrometry, and QSAR techniques. J. Pharm. Biomed. Anal., 187 (2020) 113352. https://doi.org/10.1016/j.jpba.2020.113352

[8] International Conference on Harmonization. Guideline Q8 (R2). 2017.

[9] Jankovic A, Chaudhary G, Goia F. Designing the design of experiments (DOE) – An investigation on the influence of different factorial designs on the characterization of complex systems. Energy Build. 250 (2021) 111298. https://doi.org/10.1016/j.enbuild.2021.111298

[10] Lamberti F, Mazzariol C, Spolaore F, Ceccato R, Salmaso L, Gross S. Design of Experiment: A Rational and Still Unexplored Approach to Inorganic Materials' Synthesis. Sustain. Chem, 3(1) (2022) 114–130. https://doi.org/10.3390/suschem3010009

[11] Saha P, Pandey MM. Design of Experiment (DoE)-Approach Based RP-HPLC Analytical Method Development and Validation for Estimation of Efavirenz in Bulk and Formulations. J. Chromatogr. Sci., 60(1) (2022) 35–44. https://doi.org/10.1093/chromsci/bmab029

[12] Manranjan VC, Yadav DS, Jogia HA, Chauhan PL. Design of experiment (DOE) utilization to develop a simple and robust reversed-phase HPLC technique for related substances' estimation of omeprazole formulations. Sci. Pharm., 81(4) (2013) 1043–1056. http://dx.doi.org/10.3797/scipharm.1306-06

[13] Kumar L, Sreenivasa Reddy M, Managuli RS, Pai KG. Full factorial design for optimization, development and validation of HPLC method to determine valsartan in nanoparticles. Saudi Pharm. J., 23(5) (2015) 549–555. http://dx.doi.org/10.1016/j.jsps.2015.02.001

[14] Arumugam A, Joshi A, Vasu KK. Development and Validation of a Stability-Indicating HPLC Method for Imidapril and Its Degradation Products Using a Design of Experiment (DoE) Approach. J. AOAC. Int. 100 (2017) 1727–1738. DOI: 10.5740/jaoacint.16-0329

[15] Kayesh R, Sultan MZ, Rahman A, Uddin MG, Aktar F, Rashid MA. Development and Validation of a RP-HPLC Method for the Quantification of Omeprazole in Pharmaceutical Dosage Form. J. Sci. Res., 5(2) (2013) 335–342. http://dx.doi.org/10.3329/jsr.v5i2.12779

[16] Prajapati P, Shahi A, Acharya A, Shakar Puluso V, Shah S. Implementation of White Analytical Chemistry–Assisted Analytical Quality by Design Approach to Green Liquid Chromatographic Method for Concomitant Analysis of Anti-Hypertensive Drugs in Human Plasma. J. Chromatogr. Sci., bmad054 (2023). https://doi.org/10.1093/chromsci/bmad054

[17] Prajapati P, Patel A, Shah S. Simultaneous Estimation of Telmisartan, Chlorthalidone, Amlodipine Besylate and Atorvastatin by RP-HPLC Method for Synchronous Assay of Multiple FDC Products Using Analytical FMCEA-Based AQbD Approach. J. Chromatogr. Sci., 61 (2) (2023), 160-171. https://doi.org/10.1093/chromsci/bmac030

[18] Díaz EG, de Mecca MM, Castro JA. Reactions of Nifurtimox with critical sulfhydryl-containing biomolecules: Their potential toxicology relevance. J. Appl. Toxicol. 24(3) (2004) 189–195. DOI: 10.1002/jat.970

[19] Paulos C, Paredes J, Vasquez I, Kunze G, Gonzalez-Martin G. High-performance liquid chromatographic determination of nifurtimox in human serum. J. Chromatogr., 433 (1988), 359–362.

[20] Padró JM, Marsón ME, Mastrantonio GE, Altcheh J, García-Bournissen F, Reta M. Development of an ionic liquid-based dispersive liquid-liquid microextraction method for the determination of nifurtimox and benznidazole in human plasma. Talanta, 107 (2013), 95–102. http://dx.doi.org/10.1016/j.talanta.2012.12.050

[21] Gonzalez-Martin G, Thambo S, Paulos C, Vfisquez I, Paredes J. The pharmacokinetics of nifurtimox in chronic renal failure. Eur. J. Clin. Pharmacol. 42 (1992) 671–674.

[22] International Conference on Harmonization. Guideline Q2(R1). 2005.

[23] Zhong X, Lv Q, Yong Q, Hu W, Li D, Ji S, Zhan, L, Chen, W, Li, M, Lin, J, Sun, G. Forced degradation studies of elagolix sodium with the implementation of high resolution LC-UV-PDA-MSn (n = 1,2,3…) and NMR structural elucidation. J. Pharm. Biomed. Anal., 224 (2023) 115198. https://doi.org/10.1016/j.jpba.2022.115198

[24] Alvarenga Junior BR, Lajarim Carneiro R. Chemometrics Approaches in Forced Degradation Studies of Pharmaceutical Drugs. Molecules. 24 (2019), 3804. doi:10.3390/molecules24203804

[25] Biošić M, Škorić I, Beganović J, Babić S. Nitrofurantoin hydrolytic degradation in the environment. Chemosphere,186 (2017) 660–8. http://dx.doi.org/10.1016/j.chemosphere.2017.08.01

[26] Szabó-Bárdos E, Cafuta A, Hegedűs P, Fónagy O, Kiss G, Babić S, Škorićd, I, Horvátha,O. Photolytic and photocatalytic degradation of nitrofurantoin and its photohydrolytic products. J. Photochem. Photobiol. A. Chem., 386 (2020).. 112093. https://doi.org/10.1016/j.jphotochem.2019.112093

[27] Hou Y, Yuan G, Wang S, Yu Z, Qin S, Tu L, Yan, Y, Chen, X, Zhu, H, Tang, Y. Nitrofurazone degradation in the self-biased bio-photoelectrochemical system: g-C3N4/CdS photocathode characterization, degradation performance, mechanism and pathways. J. Hazard Mater., 384 (2020), 121438. https://doi.org/10.1016/j.jhazmat.2019.121438

[28] Tauber V, Chiurciu V. Forced degradation of metronidazole, oxytetracycline and furazolidone studies using High Performance Liquid Chromatography. Veterinary Drug, 8(1) (2014), 73–79.

[29] Montgomerty D. Using fractional factorial designs for robust process development. Quality Engineering, 3(2), (1990), 193-205, DOI: 10.1080/08982119008918849

[30] Bhattacharya S. "Central Composite Design for Response Surface Methodology and Its Application in Pharmacy". Response Surface Methodology in Engineering Science, edited by Pnikumar Kayaroganam. IntechOpen, (2021). doi: 10.5772/intechopen.95835

[31] Atkinson AC, Riani M, Corbellini A. The analysis of transformations for profit-and-loss data. Appl. Statist., (2020), 0035–9254/20/69000

[32] Riani M, Atkinson AC, Corbellini A. Automatic robust Box–Cox and extended Yeo–Johnson transformations in regression. Stat. Methods Appt., 32(1) (2023) 75–102. https://doi.org/10.1007/s10260-022-00640-7

[33] Uyen VTN, Son NH. Improving accuracy of surface roughness model while turning 9XC steel using a Titanium Nitride-coated cutting tool with Johnson and Box-Cox transformation. AIMS Mater Sci., 8(1) (2021) 1–17. DOI: 10.3934/matersci.2021001

[34] Trung DD, Ngoc ND, Hong TT, Van Khoa V, Tu NT, Giang TN, Dung, NTQ, Pi, VN. Influences of Cutting Parameters on Surface Roughness During Milling and Development of Roughness Model Using Johnson Transformation. Lect. Notes Netw., 178 (2021), 491–500. https://doi.org/10.1007/978-3-030-64719-3_55

[35] Arumugam A, Joshi A, Vasu KK. Development and Validation of a Stability-Indicating HPLC Method for Imidapril and Its Degradation Products Using a Design of Experiment (DoE) Approach. J. AOAC Int., 100(6) (2017), 1727–1738. DOI: 10.5740/jaoacint.16-0329

Author statement - **MOLLIQ-D-23-08327**

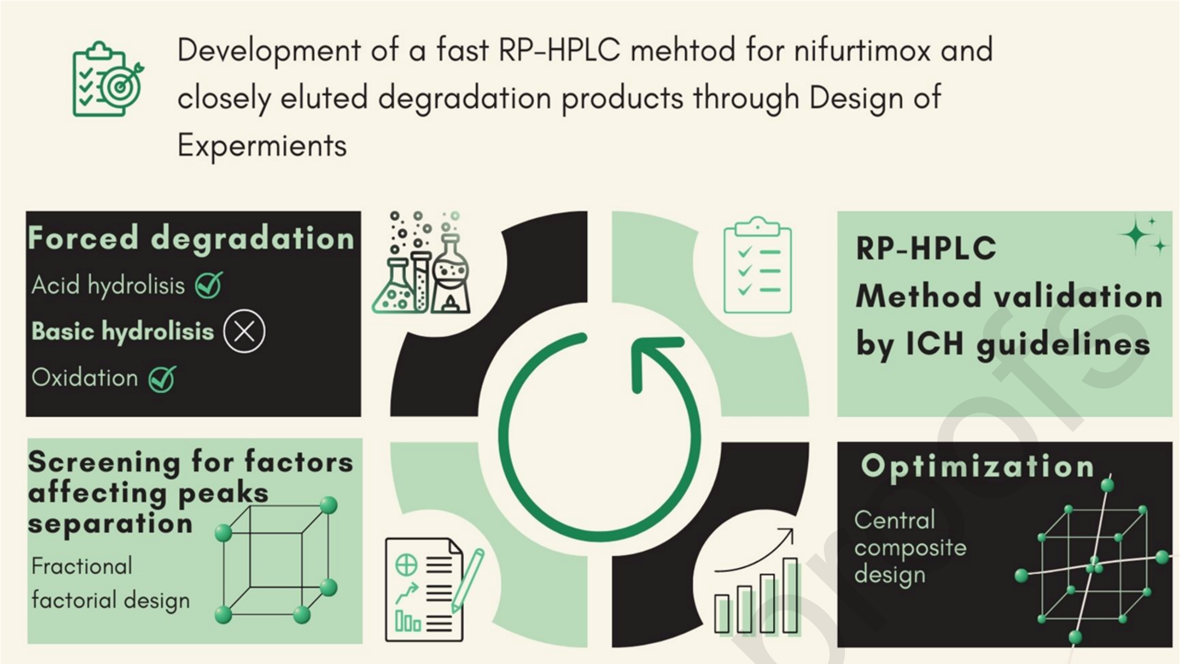
The work described in the submitted manuscript has not been published previously, it is not under consideration for publication elsewhere, and its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. If accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

During the preparation of this work, no generative AI and AI-assisted technologies have been used by the authors.

This revised version of the manuscript has been read and approved by all the authors.

Highlights

- Forced degradation products of nifurtimox were generated by acidic and basic hydrolysis
- A Design of Experiments approach was applied to evaluated how different chromatographic conditions affected resolution, retention time and tailing factor
- A fast RP-HPLC method for separation of nifurtimox and closely eluted degradation products was developed
- The developed RP-HPLC method was validated as indicated by ICH guidelines



Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Carlos Boschetti reports financial support was provided by Santa Fe Agency of Science Technology and Innovation. Claudio Salomon reports financial support was provided by Argentina Ministry of Science Technology and Innovation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.