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TECHNICAL NOTE

Risk-Based Selection of Active Pharmaceutical Ingredients in the Development of HPLC and NIR Methods for Pediatric Preparations

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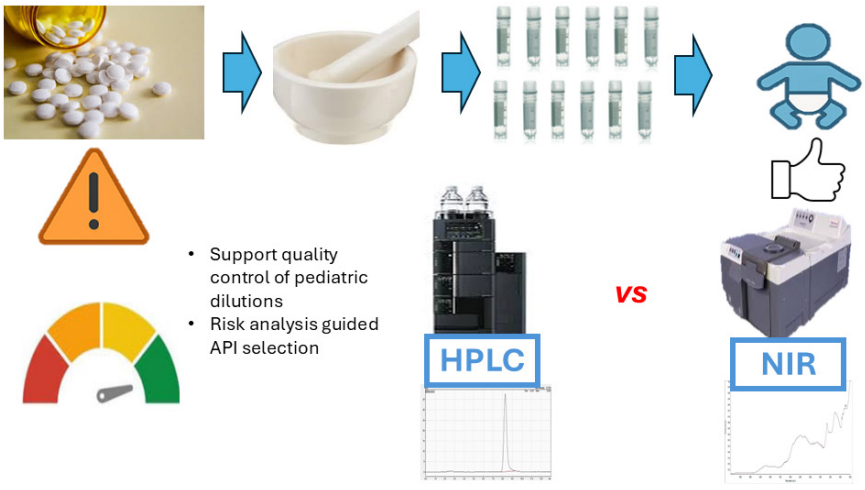

The lack of appropriate formulations for the pediatric population often requires the manipulation of adult dosage forms in hospital settings, a practice that can lead to errors that impact patient safety. This study aimed to support the quality control of pediatric preparations by developing and validating analytical methods for the quantification of active pharmaceutical ingredients (APIs) in the tablets used for their elaboration. Folic acid and phenobarbital were selected based on a risk analysis as the priority APIs to work with. High-performance liquid chromatography (HPLC) and Near-infrared (NIR) spectroscopy methods for the quantification of these APIs in commercially available products were developed and validated. NIR methods are presented as a rapid and non-destructive alternative that offers the possibility of determining the content of the same tablets that will be used to prepare the pediatric dilution. This could be considered as a promising analytical tool for drug quality control during the elaboration process of these preparations.

Keywords: Near-infrared spectroscopy, pediatric preparations, folic acid, phenobarbital, drug quality control

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INTRODUCTION

There is a global shortage of medicines suitable for the pediatric population, as the pharmaceutical industry has conducted limited research in this area due to technical, economic, and ethical reasons.[1-3] As a result, there are relatively few formulations appropriate for children,[1] often leading to use of medications formulated for adults outside their approved indications (off-label) or their manipulation (e.g., splitting or crushing tablets) to obtain suitable pediatric doses.[4]

Off-label use is widespread, with reported prevalence ranging from 3.3% to 94%, with the highest rates commonly observed in neonatal clinical care settings.[3] A common practice in hospital settings is the preparation of solid dilutions from adult medications. At the Pharmacy Department of the *Hospital de Clínicas* (University Hospital, Montevideo, Uruguay), for instance, pediatric dilutions are routinely prepared to treat preterm and neonatal patients at the institution. These dilutions are made by grinding tablets in a mortar and mixing them with an excipient, such as lactose, before being divided into vials. Errors in this process—such as improper mixing or segregation of the blend during portioning—can lead to dose non-uniformity and result in inadequate treatment. This risk is heightened by the absence of standardized guidelines or procedures for these preparations.[5]

These circumstances highlight the need to study and standardize the processes of elaboration to ensure the safety and efficacy of pediatric treatments. The objective, therefore, is to ensure the quality and assess the uniformity of the prepared pediatric dilutions, as well as to determine their shelf life. Achieving this goal requires suitable analytical methods for quantifying the active pharmaceutical ingredient in the initial dosage form and its dilution.

Near-infrared spectroscopy (NIR) is proposed as a rapid and non-destructive technique with various applications in the pharmaceutical industry reported, including identification tests, quantification, and process monitoring.[6] NIR can be applied to analyze both the source tablets and the corresponding dilutions once prepared. Analyzing the source tablets will ensure the selection of the most suitable for pediatric preparation. This is particularly relevant considering that, according to the acceptance criteria for the Uniformity of Dosage Units test, tablet content may vary approximately between 75% to 125% of the labeled amount,[7] potentially impacting the accuracy of the prepared doses.

MATERIALS AND METHODS

Instruments

High-performance liquid chromatography (HPLC) analyses were conducted using an Agilent 1100 HPLC system equipped with a variable wavelength UV detector. Near-infrared (NIR) spectroscopy was performed using a Thermo Scientific Antaris II DR analyzer, operated with Result 4.5.159 and TQ Analyst 9.12.116 software for data acquisition and analysis.

Reagents and chemicals

For HPLC method development, folic acid (90.54%, Sigma, Germany), phenobarbital (99.7%, Fármaco Uruguayo, Uruguay) secondary standards were used. Other reagents were of analytical grade, and methanol was of HPLC gradient grade. Type II purified water was obtained by distillation in the laboratory. For NIR analysis, no additional reagents were required, as measurements were performed on intact tablets.

Four products from different suppliers of folic acid tablets available on the Uruguayan market (two containing 1 mg and two containing 0.8 mg of folic acid) were analyzed, along with the only available local product containing 100 mg of phenobarbital, which is used by the *Hospital de Clínicas* Pharmacy Service.

Survey for pediatric dilutions

A survey at the *Hospital de Clínicas* Pharmacy Service to obtain records of the prepared pediatric solid dilutions from the period between 2012 and 2022 was conducted. Folic acid and phenobarbital emerged as the most frequently used APIs.

Telephone consultations with hospital pharmacies across Uruguay revealed varied practices. Many hospitals, lacking pediatric units, did not prepare dilutions, while others outsourced to a particular laboratory, where hydrocortisone, captopril, and clonidine were prevalent.

The survey revealed distinct API needs, *Hospital de Clínicas* prioritizing folic acid and phenobarbital, while in other institutions hydrocortisone and other APIs prevailed.

Risk analysis

To select the active ingredients to be studied, a risk analysis was conducted to prioritize those active ingredients whose study could have the greatest impact. The analysis was restricted to data from the *Hospital de Clínicas*, as data from this service apply to a small subgroup of the high-risk pediatric population, such as newborn and preterm patients.

To prioritize APIs for method development, a risk analysis was carried out, based on the principles of failure mode and effects analysis (FMEA).[8,9] This kind of analysis is based on prior knowledge, the probability of failures occurring in a manufacturing process that is the same for all active ingredients, the severity of the lack of quality in solid dilutions according to the active ingredient, and the ability to detect failures if they occur. The risk factors associated with each API were identified to determine the parameters.

Probability of failure

Probability of failure was assessed based on three factors:

1. **Preparation frequency:** APIs prepared more frequently (e.g., daily vs. monthly) were assigned higher scores due to increased opportunities for errors. In this way, a value was assigned to each API according to Table I.

Table I. Scores associated with preparation frequencies

Preparation frequency	Score
Monthly or less	10
Between monthly and annual	5
Annual or higher	1

2. **Dilution level:** higher dilutions increased the risk of non-uniform mixing, increasing the probability of failure. Based on the highest dilution level possible for each API, a score was assigned according to Table II.

Table II. Scores associated with dilution level

Dilution level	Score
Higher than 1:200	10
1:100 – 1:200	7
1:50 – 1:100	5
1:10 – 1:50	3
1:1 – 1:10	1

3. **API environmental sensitivity:** during the compounding process, APIs will be exposed to environmental conditions. These conditions can affect the API differently, depending on its sensitivity. The more sensitive the API, the greater the likelihood of failure. Storage conditions recommended by recognized suppliers were considered to assess whether an active ingredient is sensitive to temperature, humidity, and light in each case. Each condition was assessed independently by assigning a score of 2 if the API is sensitive to the environmental condition and 1 otherwise. Finally, the sensitivity to the environmental conditions was calculated as the product of the scores for each condition.

Each factor was scored independently, averaged, and normalized to a 1–10 scale.

Severity of failure

Severity was evaluated using two pharmacological parameters:

1. **Minimum daily dose:** APIs with lower minimum doses indicates that a dosage error is likely to have an adverse effect on the patient. Thus, a score was assigned to each API according to Table III.

Table III. Scores associated with minimum daily dose

Minimum daily dose	Score
(1 – 10) mg	10
(10 – 100) mg	5
Higher than 100 mg	1

2. **Lethal dose (LD50):** to assess the toxicity of each API, data on the lethal dose 50 in rats is evaluated. In cases where this information is unavailable, the values obtained for mice are considered. The use of the values obtained in mice is considered not to affect the evaluation, thus the wide ranges used to assign scores. Considering that substances are more toxic at lower lethal doses 50, scores were assigned for the different active ingredients according to Table IV.

Table IV. Scores associated with lethal dose 50

LD50	Score
Less than 200 mg kg^{-1}	10
(20 – 2000) mg kg^{-1}	5
More than 200 mg kg^{-1}	1

These parameters were averaged and normalized to a 1–10 scale.

Detectability of failure

As no analytical controls were routinely applied during dilution preparation, all APIs received the maximum score of 10, reflecting the absence of methods to detect errors during the elaboration process.

From the values obtained, the risk priority number (RPN) is calculated as the product of probability, severity and detectability.

The values obtained will allow professionals to reach a decision on which active ingredients are prioritized for work. The results were classified as presented in Table V.

Table V. Classification of priorities according to RPN values

RPN	Score
Less than 100	Low priority
100 – 200	Medium priority
Higher than 200	High priority

HPLC method development

Folic acid

The HPLC method for folic acid quantification in tablets was developed with a UV detector set at 280 nm. Chromatographic conditions were optimized through a central composite design. Based on prior knowledge, critical methods attributes (CMAs), proportion of organic solvent (8%–12% v/v methanol), column temperature (20 ºC–40 °C), and mobile phase pH (5.6–7.2), were selected to evaluate their impact on the critical quality attributes (CQAs) defined for the method: retention time, resolution between folic acid and leucovorin, and peak symmetry. The effects of the studied factors on the defined CQAs were first evaluated using the regression coefficients and their statistical significance (p-values <0,05) (Table VI), together with the standardized effect estimates with their 95% confidence intervals, as shown in Figure 1.

Table VI. Regression coefficients and their p-value in folic acid HPLC method development

	Peak Symmetry		Resolution		Retention Time	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
Buffer pH (pH)	0,0303	0,0442	-0,1554	<0,0001	-1,5490	<0,0001
Methanol Content (%MeOH)	0,0498	0,0050	-0,1509	<0,0001	-1,2513	<0,0001
Temperature (T)	0,0750	0,0005	-0,1839	<0,0001	-0,3413	0,0003
pH*pH	-0,0124	0,6188	0,0716	<0,0001	0,3577	0,0084
%MeOH*%MeOH	0,0211	0,4076	0,0164	<0,0001	0,1742	0,1205
T*T	0,0131	0,6019	0,0224	<0,0001	-0,1488	0,1750
pH*%MeOH	0,0143	0,3373	0,0252	<0,0001	0,4224	0,0001
pH*T	0,0278	0,0849	0,0150	<0,0001	-0,1821	0,0152
%MeOH*T	0,0275	0,0872	0,0269	<0,0001	-0,0074	0,9008

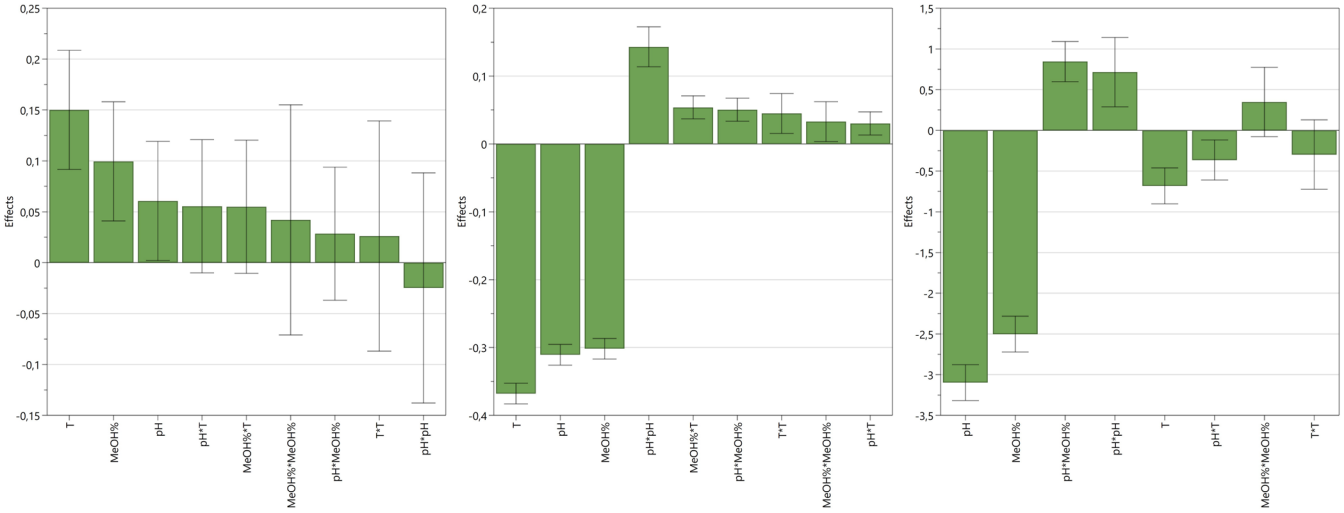

Figure 1. Standardized effects plots obtained for CQAs in folic acid HPLC method development.

A predictive model was then constructed using Multiple Lineal Regression (MLR), excluding non-significant factors (p-values <0,05). Model was assessed through the determination coefficients (R^2) and predictive ability (Q^2) presented in Table VII. The model presents good correlation and strong predictive ability for retention time and number of theoretical plates, while peak symmetry showed moderate correlation and acceptable predictive power.

Table VII. Regression model parameters for the CQAs in folic acid HPLC method development

CQA	R^2	Q^2
Peak Symmetry (Tailing)	0.769	0.560
Resolution	0.999	0.984
Retention time	0.993	0.977

Finally, response contour plots were generated to visualize the effect of the most influential factors and their interactions on the chromatographic performance (Figure 2). The analysis showed that retention time was affected by methanol content, pH and temperature, while resolution was strongly dependent on methanol content and pH. Peak symmetry proved relatively robust with only minor contributions from temperature and methanol content.

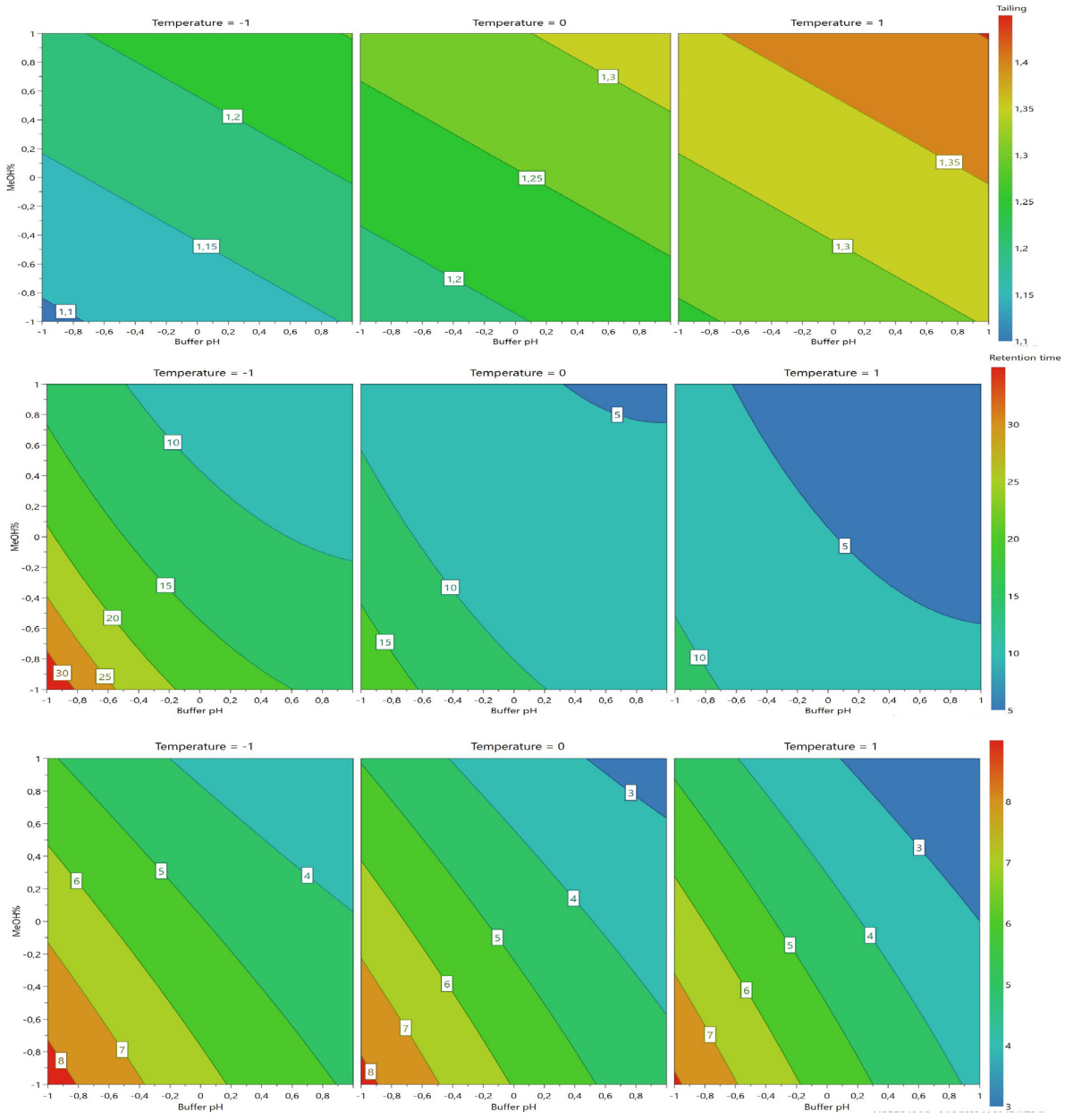

Figure 2. Response contour plots of the factor on the CQAs in folic acid method development.

The optimized conditions were: Zorbax Eclipse XDB C18 column (250 mm × 4.6 mm, 5 µm), mobile phase of phosphate buffer (pH 6.4):methanol (90:10), column temperature of 30 °C, flow rate of 1.0 mL min^{-1}, and injection volume of 25 µL.

Sample preparation involved placing one tablet in a 100.00 mL volumetric flask, adding 2.5 mL of sodium carbonate solution (28 g L^{-1}) and 50.0 mL of purified water, and agitation for 20 minutes using an orbital shaker. The solution was brought to volume with purified water, homogenized in an ultrasonic bath, allowed to settle, and filtered through 0.45 µm cellulose filters. A 10 mg L^{-1} standard solution of folic acid was prepared in purified water with the same volume of sodium carbonate solution added to aid dissolution.

Phenobarbital

The HPLC method for phenobarbital quantification was developed with a UV detector set at 254 nm. A fractional factorial screening design of resolution IV, to evaluate the impact of potentials CMAs like pH (3.8–5.0) and ionic strength (50 mM–200 mM) of the buffer component of the mobile phase, as well as methanol percentage (30%–70%), column temperature (25 ºC–35 °C), flow rate (0.6 mL min^{-1}–1.2 mL min^{-1}), and injection volume (10 µL–50 µL), on CQAs defined for the method: retention time, number of theoretical plates, and peak symmetry. As for phenobarbital, the influence of the studied factors on the selected CQAs was initially assessed by examining the regression coefficients and their statistical significance (p-values <0,05) (Table VIII), together with the standardized effect estimates with their 95% confidence intervals, as shown in Figure 3.

Table VIII. Regression coefficients and their p-value in phenobarbital HPLC method development

	Peak Symmetry		Number of Theoretical Plates		Retention Time	
	Coefficient	**p-value**	**Coefficient**	**p-value**	**Coefficient**	**p-value**
pH Buffer	-0,0019	0,8884	-360,69	0,3881	-0,8085	0,6963
MeOH%	0,0779	0,0001	-2583,44	<0,0001	-15,3244	<0,0001
Temperature	-0,0091	0,5003	1590,81	0,0019	-1,4254	0,4943
Ionic Strength	-0,0075	0,5785	3,5639	0,9931	-0,9416	0,6497
Flow	0,0066	0,6231	-2767,44	<0,0001	-6,1285	0,0104
Injection Volume	-0,0070	0,6038	642,81	0,1364	0,0916	0,9646

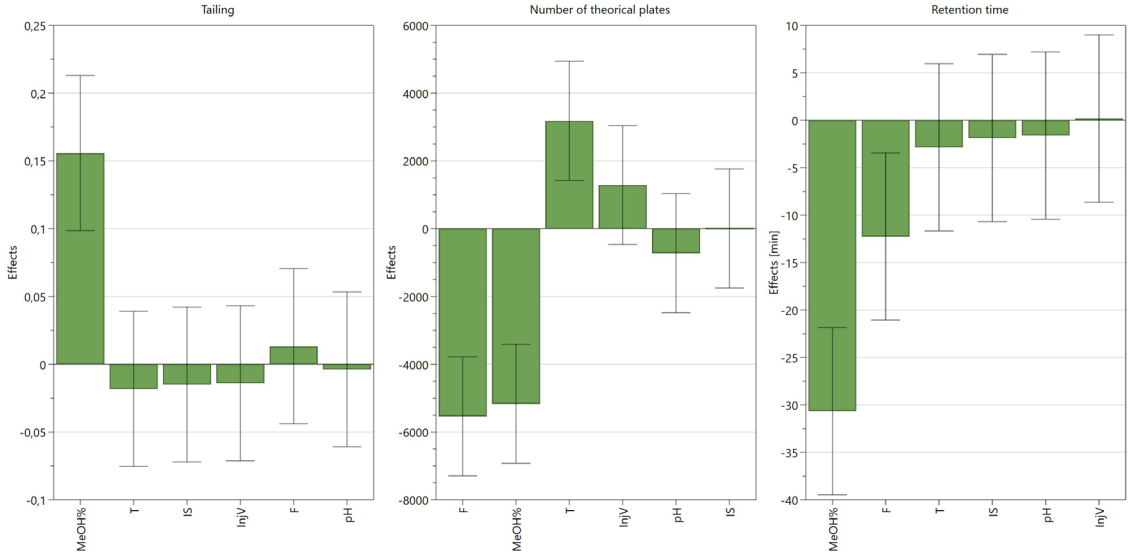

Figure 3. Standardized effects plots obtained for CQAs in phenobarbital HPLC method development.

A MLR model was built, excluding non-significant factors. As shown in Table IX, the model achieved good correlation and predictive ability for retention time and number of theoretical plates, while peak symmetry displayed moderate correlation and predictive power.

Table IX. Regression model parameters for the CQAs in phenobarbital method development

CQA	R2	Q2
Peak Symmetry (Tailing)	0.725	0.656
Number of Theoretical Plates	0.925	0.850
Retention time	0.909	0.879

Contour plots were generated to offer a graphical interpretation of the influence of the key factors and their interactions on the CQAs (Figure 4). The methanol proportion was identified as the most influential factor, simultaneously reducing retention time, decreasing efficiency, and increasing peak tailing. Flow rate also had a marked effect on retention time, while higher temperatures improved efficiency. Buffer pH and ionic strength contributed mainly to column efficiency, whereas peak symmetry proved relatively robust, showing only minor sensitivity to column temperature and injection volume.

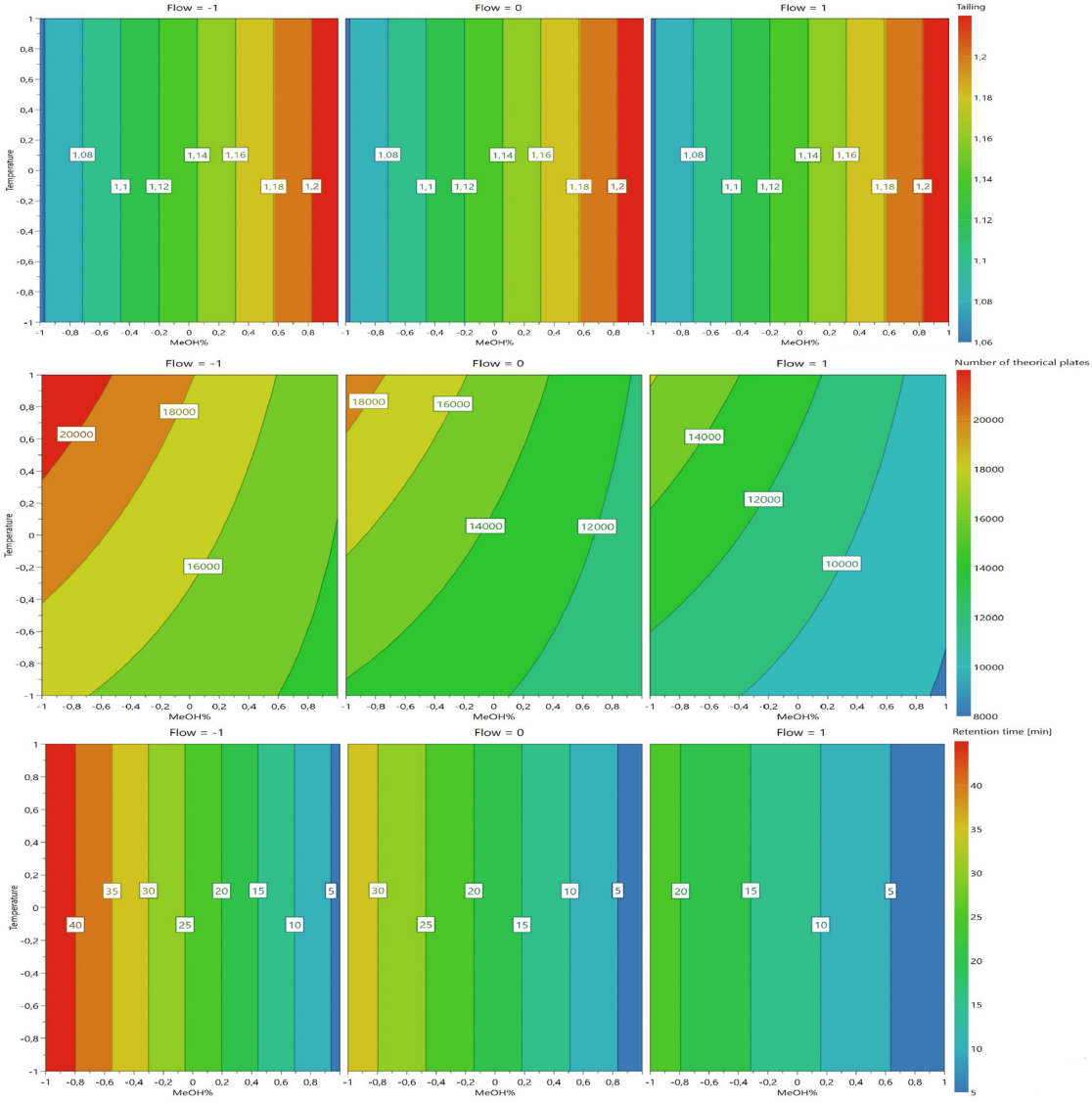

Figure 4. Response surface contour plots of the factor on the CQAs in phenobarbital method development.

The final method conditions were: Waters Symmetry C18 column (250 mm × 4.6 mm, 5 µm), mobile phase of 200 mM acetate buffer (pH 3.8): methanol (60:40), column temperature of 35 °C, flow rate of 1.0 mL min^{-1}, and injection volume of 35 µL.

Sample preparation involved placing one tablet in a 100.00 mL volumetric flask, adding 50.0 mL of purified water, and agitation for 20 minutes on an orbital shaker. The solution was brought to volume with purified water, homogenized in an ultrasonic bath, and allowed to settle. A 5.00 mL aliquot was diluted to 50.00 mL with purified water and filtered through 0.45 µm cellulose filters. A 10 mg L^{-1} standard solution of phenobarbital prepared in purified water.

NIR method development

Calibration sets consisted of 30 tablets per product, analyzed by both HPLC (as the reference method) and NIR. Tablets were scanned in diffuse reflectance mode over the 4000–10000 cm^{-1} range, with 32 scans averaged per spectrum at a resolution of 8 cm^{-1}. A calibration model was built using Partial Least Squares (PLS) regression to correlate NIR spectra with API concentrations determined by HPLC, optimizing the number of PLS factors to minimize the root mean square error of calibration (RMSEC).[10] Pre-processing techniques, including standard normal variate (SNV) transformation and first-derivative smoothing, were applied to reduce baseline shifts and enhance spectral features.[11] Figure 5 shows a NIR spectrum of folic acid tablets from Product 1, while Figure 6 presents the corresponding spectrum for phenobarbital tablets.

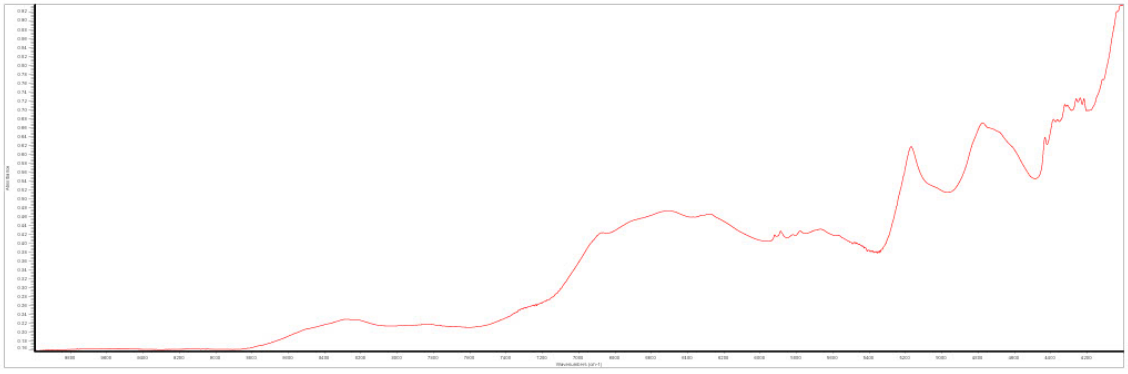

Figure 5. NIR spectrum of folic acid tablets from Product 1.

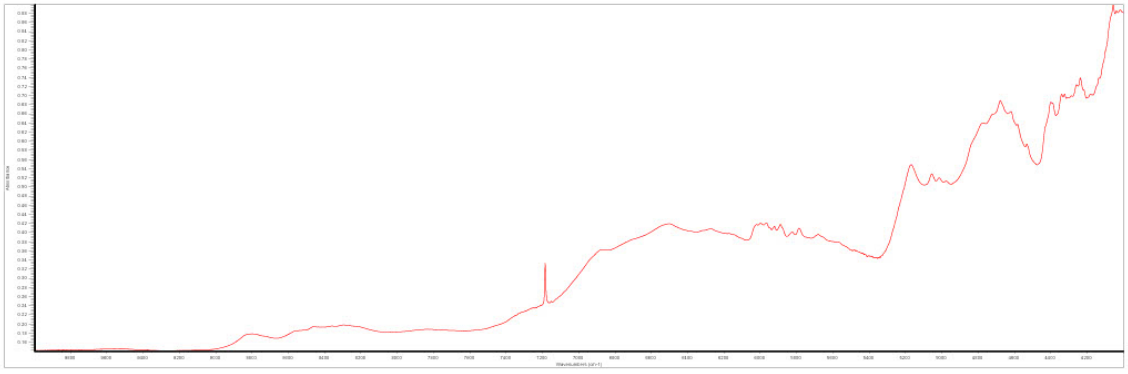

Figure 6. NIR spectrum of phenobarbital tablets.

Method validation

The analytical procedure validation was performed following the recommendations of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2 guidelines.[12] The performance characteristics evaluated for both HPLC methods were accuracy, repeatability, linear response and specificity.

Validation of the novel proposed methodology using NIR was conducted with an independent set of 10 tablets per API, scanned under the same conditions as the calibration set. The performance characteristics evaluated were accuracy and repeatability.

RESULTS AND DISCUSSION

Survey and risk analysis

The survey identified folic acid and phenobarbital as the most frequently used APIs at the *Hospital de Clínicas*, primarily for neonates and preterm infants, with lactose as the diluent excipient. Other institutions reported hydrocortisone, captopril, and others, reflecting different pediatric subgroups. The FMEA results (Table X) confirmed folic acid and phenobarbital as critical APIs. Folic acid's high RPN resulted from frequent preparation, low therapeutic dose, and poor detectability, while phenobarbital's RPN reflected frequent use, high dilution levels, and a narrower therapeutic window. The risk-based approach ensured that method development targeted APIs with the greatest potential impact on patient safety, particularly for vulnerable neonates.

Based on the risk factors considered in this analysis, the control strategy prioritized the development of analytical methods to enhance the detectability of potential quality deviations.

Table X. Risk Analysis Summary for Selected APIs at Hospital de Clínicas

API	Probability	Severity	Risk	Detectability	RPN
Amiodarone	2	1	2	10	20
Captopril	6	3	17	10	165
Chlorpromazine	4	8	30	10	300
Ciprofloxacin	2	3	6	10	60
Fluconazole	2	3	6	10	60
Folic Acid	8	6	41	10	413
Furosemide	2	3	6	10	60
Hydrochlorothiazide	2	3	6	10	60
Hydrocortisone	3	3	9	10	90
Levetiracetam	1	1	1	10	10
Levothyroxine	1	10	10	10	100
Leucovorin	1	3	3	10	30
Nelfinavir	2	1	2	10	20
Nevirapine	2	1	2	10	20
Phenobarbital	5	8	38	10	375
Phenytoin	2	3	6	10	60

(continues on next page)

Table X. Risk Analysis Summary for Selected APIs at Hospital de Clínicas (continuation)

API	Probability	Severity	Risk	Detectability	RPN
Pyridostigmine	2	6	11	10	110
Pyrimethamine	2	5	10	10	100
Propranolol	3	3	9	10	90
Sildenafil	2	5	10	10	100
Spironolactone	2	5	10	10	100
Sucralfate	3	1	3	10	30
Sulfadiazine	2	1	2	10	20
Ursodeoxycholic Acid	2	1	2	10	20
Valganciclovir	2	1	2	10	20
Vigabatrin	2	1	2	10	20
Vitamin B1	2	3	6	10	60

HPLC method

The HPLC methods for folic acid and phenobarbital were validated according to the ICH guidelines.[12] Evaluation of linear response included visual inspection of the calibration curves, assessment of the determination coefficient (R^2), and confirmation of homoscedasticity and linearity of residuals. Linear response was achieved over 25–150 mg L^{-1} for phenobarbital and over 2.5–15 mg L^{-1} for folic acid. The calibration functions were $y = 9.00\times10^{7}\cdot x + 801$ ($R^2 = 0.9997$) for folic acid and $y = 1.11\times10^{7}\cdot x + 4.35\times10^{3}$ ($R^2 = 0.9998$) for phenobarbital where *y* was the peak area and *x* was the analyte concentration in mg mL^{-1}.

Specificity was verified through forced degradation studies. For folic acid, 5.0 mL of a 1.0 mg mL^{-1} solution were exposed to the conditions presented in Table XI, while for phenobarbital, 5.0 mL of a 0.5 mg mL^{-1} solution were exposed to the conditions presented in Table XII. The percentage of degradation observed under each condition is summarized in Tables XI and XII. This percentage was calculated as the difference in API content measured in the stressed solution compared to an unstressed reference solution. Folic acid exhibited significant degradation to oxidative and photolytic stress highlighting the need for light protection during handling and storage. In contrast, phenobarbital proved generally stable, showing significant degradation only under basic hydrolytic conditions, while no relevant degradation was observed under the other stress conditions. In all conditions studied, a resolution greater than 2 was obtained for both analytes in relation to the main degradation products, demonstrating that both methods are stability-indicating. For folic acid, specificity was further confirmed by evaluating structurally related compounds such as p-aminobenzoic acid and leucovorin.

Table XI. Forced degradation studies of folic acid

Degradation condition	Stress details	% Degradation observed
Acidic	5 mL HCl 1 M, 1-week, room temperature	3.6
Basic	5 mL NaOH 1 M, 1-week, room temperature	5.5
Oxidative	5 mL H_2O_2 3%, 1-week, room temperature	24.6
Photolytic	Natural light, 1-week, room temperature	28.0

Table XII. Forced degradation studies of phenobarbital

Degradation condition	Stress details	% Degradation observed
Acidic	5 mL HCl 5 M, 3 h, 70 ºC	0.5
Basic	5 mL NaOH 1 M, 15 min, 70 ºC	25.1
Oxidative	5 mL H_2O_2 3% w/v, 24 h, room temperature	3.3
Photolytic	UV lamp (254 nm 15 W), 3 h, room temperature	0

Accuracy was assessed through the analysis of spiked samples, since reconstruction of the original matrix was not possible due to the lack of information on its composition.[12] Repeatability was expressed as the relative standard deviation (n=6, RSD%). Detailed results are presented in Table XIII.

Table XIII. HPLC method validation results for four products of folic acid and phenobarbital

Pharmaceutical Product	API	Content (mg)	Repeatability (RSD %, n=6)	Accuracy (% Mean Recovery)
Product 1	Folic Acid	1	0.57	99.5
Product 2	Folic Acid	0.8	0.82	99.2
Product 3	Folic Acid	1	1.3	101.4
Product 4	Folic Acid	0.8	1.6	100.8
Product 5	Phenobarbital	100	0.72	100.4

These results were considered suitable for the intended purpose.

NIR method development

Accuracy and repeatability were assessed for NIR methods. Accuracy was verified as the average value (n=10) of the difference between the NIR and HPLC value results, using tablets that were not included in the calibration set. Repeatability was expressed as the relative standard deviation (n=10, RSD%) of 10 determinations of the same tablet. Detailed validation results are presented in Table XIV.

Table XIV. NIR method validation results for four suppliers of folic acid and one of phenobarbital

Pharmaceutical Product	API	Content (mg)	R^2	RMSEC	PLS factors	Repeatability (RSD %, n=6)	Deviation from HPLC results (%)
Product 1	Folic Acid	1	0.9988	0.0627	6	1.7	0.44
Product 2	Folic Acid	0.8	0.9996	0.0538	4	1.4	0.65
Product 3	Folic Acid	1	0.9960	0.243	4	2.7	1.9
Product 4	Folic Acid	0.8	0.9994	0.224	10	2.2	1.0
Product 5	Phenobarbital	100	0.9677	1.12	7	0.74	1.8

Based on these results, the methods provided a rapid, non-destructive alternative to HPLC, with potential for improved calibration through additional samples.

CONCLUSIONS

Through a risk analysis approach the priority for developing analytical methods for folic acid and phenobarbital was demonstrated.

A novel and rapid, non-destructive NIR analytical method was developed to determine the concentration of the active ingredients of interest in those tablets that will be used to prepare pediatric dilutions. Results showed that NIR can provide reliable results. This approach provided an effective control strategy for improving the detectability of quality deviations. Future work will focus on validating HPLC and NIR methods for the quantification of these APIs directly in the pediatric dilutions prepared at the Pharmacy Department and on evaluating their stability.

This research addresses a real problem that still has no solution for the on-demand production of a product aimed at a vulnerable population that is currently prepared by hand without enough guarantee regarding the final dose of the active ingredient.

Conflicts of interest

Authors declare no conflicts of interest.

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