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Original research paper

HPLC method development for fampridine using Analytical Quality by Design approach

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Offering a systematic and multivariate analysis of the analytical procedure, development and validation of HPLC methods using Quality by Design approach are in the limelight of current research trends. A new, experimental design-aided HPLC method for fampridine was developed and preliminarily validated according to current in-force international guidelines for linearity, accuracy, robustness and precision.

The method offers a high throughput sample analysis, with an elution time of 2.9 minutes, and signal detection without excipient interference performed at 262 nm. The method proved to be linear between 1–15 μg mL^{-1} (R^2 = 0.9996). The mean recovery was found to be 98.7 ± 1.9 % in the tested range of 2.5–7.5 μg mL^{-1}. Low RSD values (< 1 %) were obtained for both model, intra- and inter-day precision. The limit of detection and limit of quantification were 0.24 and 0.78 μg mL^{-1}, resp. The method proved to be applicable for active substance assay in a pharmaceutical dosage form.

Keywords: Analytical Quality by Design, fampridine, HPLC

Multiple sclerosis (MS) is a chronic neuroinflammatory disease that affects approximately 2.5 million people worldwide. Genetic susceptibility and environmental factors are

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probably involved in the onset of the disease, however, the exact cause of MS is still unknown (1). Fampridine (4-aminopyridine, dalfampridine, Fig. 1) is a recently developed therapeutic agent, that complements the currently available disease-modifying treatments by ameliorating the walking impairment, a symptom with high prevalence among MS patients (2). Fampridine is a broad-spectrum voltage-dependent potassium channel blocker first used in electrophysiological studies to determine the role of potassium channels in the generation and propagation of neuronal action potentials (3, 4). Following two successful phase-3 clinical trials (5, 6), the US Food and Drug Administration (FDA, 2010) and the European Medicines Agency (EMA, 2011) approved fampridine for the management of walking disability in patients with MS (7, 8).

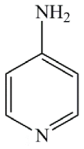

Fig. 1. Chemical structure of fampridine (4-aminopyridine).

A limited number of analytical methods have been developed for the assay of fampridine. Determination of the active substance and related impurities in the bulk drug or formulations has been achieved using UV spectrophotometry (9) or high-performance liquid chromatography (HPLC) (10–13). Furthermore, liquid chromatography/mass spectrometry (LC-MS) procedures have been developed and applied for the assay of fampridine in biological samples, either individually (14) or simultaneously with other active substances (15, 16).

The United States Pharmacopeia's (USP) monograph for dalfampridine (US name for fampridine) describes an HPLC assay procedure that uses as mobile phase methanol and a buffer solution containing octanesulfonic acid sodium salt, ammonium acetate and trimethylamine (17). Thomas *et al.* (10) used a mobile phase (pH = 4.00 ± 0.05) of similar composition for the determination of fampridine and potential impurities in the bulk drug. The method described by Babu *et al.* (11) employs as mobile phase A buffer solution of potassium dihydrogen phosphate and octanesulfonic acid and as mobile phase B mixture of acetonitrile and mobile phase A. Dharani *et al.* (12) have reported a simpler mobile phase composition, consisting of potassium dihydrogen phosphate solution and acetonitrile with an isocratic elution.

A design of experiments (DoE) approach supports the simultaneous variation of multiple factors at different levels, allowing the detection of both main effects and secondary interactions between the studied factors (18, 19). Therefore, DoE provides relevant information with a minimal number of performed experiments (20). Based on the results of these experiments the optimal run conditions can be statistically determined and the outcome of unperformed experiments predicted with high accuracy (21). Pharmaceutical applications of DoE include the development and optimization of single-component (22, 23) and multi-component formulations (24, 25), as well as the development and validation of analytical methods (26, 27).

The main objective of the present study was to develop and validate a high throughput HPLC method for the assay of fampridine applying DoE using readily available reagents, thus providing a simple and cost-effective alternative to available techniques. Furthermore, we examined the effectiveness of a DoE approach for method development and validation.

EXPERIMENTAL

Instrumentation

HPLC determination was carried out using a LiChrosphere® 60 RP-select B 5 μm, 250 mm × 4 mm column (Merck KGaA, Germany). The components of the Merck Hitachi HPLC system were the following: interface D-7000, quaternary pump L-7100, solvent degasser L-7612, autosampler L-7200, column oven L-7360 and DAD detector L-7455. Specific chromatograms were analysed using the HSM 4.0 software by Merck Hitachi (Merck Hitachi, Japan).

Reagents

Disodium hydrogen phosphate, monobasic potassium phosphate, orthophosphoric acid, sodium hydroxide and HPLC grade methanol (MeOH) were used from Merck (Merck). Gradient grade acetonitrile (ACN) was from VWR International (VWR International, France). Dalfampridine USP reference standard was obtained from USP (USP, Rockville, MD, USA).

The active substance fampridine (FAM) was obtained from Procos (Procos SpA, Italy). Water, purified, was obtained with a Merck Millipore Direct Q, Progard 2® system (Merck Millipore, USA).

Selectivity studies were performed based on the qualitative and quantitative composition of the original product, Fampyra 10 mg prolonged-release tablets, using Hypromellose (Dow Chemical, USA), cellulose, microcrystalline (JRS Pharma, Germany), silica, colloidal anhydrous (Evonik Industries, Germany) and magnesium stearate (Faci SpA, Italy) as excipients. The original product was purchased from a local pharmacy.

Preparation of phosphate buffer solution. – Phosphate buffer solution with pH = 6.8 was prepared according to the current USP (17).

Preparation of placebo solution (real blank). – The quantities of selected excipients corresponding to one single dose of pharmaceutical formulation were stirred using 900 mL phosphate buffer, pH = 6.8. Ten mL of the solution was filtered through a 0.45-μm Chromafil® Xtra PA-45/25 filters (Macherey-Nagel, Germany) and completed to 1 L in a volumetric flask with water, obtaining a 1:100 diluted placebo.

Preparation of standard solutions. – The solution of a final concentration of 5 μg mL^{-1} was prepared by dissolving 5 mg FAM in 1 L of 1:100 diluted placebo formulation and completed to the mark with the same solvent.

The starting solution for linearity testing was prepared by dissolving 20 mg FAM in 1 L of 1:100 diluted placebo formulation and completed to the mark with the same solvent, thus obtaining a final concentration of 20 μg mL^{-1}.

Chromatographic conditions

Chromatograms were recorded under the conditions obtained by *in silico* method development optimization, using an isocratic mobile phase consisting of MeOH:ACN:PB 65:5:30 (%, *V*/*V*) (PB – phosphate buffer 1 mmol L^{-1}, pH = 3.0 ± 0.01 adjusted with H_3PO_4). The flow rate was set at 1.0 mL min^{-1}, using a column temperature of 39.5 °C and an injection volume of 30 μL using the cut method. Analytical signal detection and best chromatogram extraction were recorded at 262 nm.

Method optimization

Screening experimental design. – The analytical method was optimized by experimental design using the MODDE 12.1 software (Sartorius Stedim Biotech GmbH, Göttingen, Germany). For the best evaluation of the chromatographic method and to profoundly investigate the effects of defined factors on selected responses a full factorial design with three center points was chosen. The included factors: flow rate, column temperature and the proportion of the phosphate buffer, pH = 3.0, were investigated at three levels, returning in 30 experiments ($3^3 = 27$ in this case, yielding a total of 30 runs including the three center, replicate runs). The level of ACN was kept at a constant ratio of 5 % throughout the method development and MeOH proportion was modified according to DoE settings for the percentage of phosphate buffer, pH = 3.0. Samples were analysed in three replicate runs, retention time, theoretical plate number and tailing factor were recorded as responses (Table I).

Model fitting and analysis of the experimental design. – The experimental model was fitted using the partial least squares (PLS) method and the significance was tested by ANOVA *F*-test and its test for lack-of-fit. Further analysis included the evaluation of model performance indicators characterizing the factor-response interactions. Generally, a model is

Table I. Factors and responses defined in the present screening experimental design

Selected factors for the experimental design			
Level applied	–1	0	1
Flow rate (mL min^{-1})	0.50	1.00	1.25
Column temperature (°C)	20	30	40
PB proportion in mobile phase (%)[a]	30	50	70
Responses followed for experimental design assessment			
Requirement	Min	Target	Max
Retention time (min)	2	3	5
Theoretical plate number (*N*)[b]	2000	–	–
Tailing factor	0.8	1.2	2.0

[a] PB – phosphate buffer, pH = 3.0.
[b] Defined per column length.

considered adequate if the goodness of fit (R^2) and goodness of predictability (Q^2) tend to 1.0, but are greater than 0.5 and the difference between the two indicators does not exceed 0.2–0.3. Model validity, describing the suitability of the selected method for the analysis of the design should return values greater than 0.25. Finally, model reproducibility offers information about the controllability of the proposed method and is considered acceptable if the values are situated above 0.5. In the case of reproducibility, excessively high numerical values (> 0.99) indicate an infinitesimal pure error inside the model and is generally observed in highly controlled methods, *e.g.*, HPLC determinations. As model validity is closely related to the pure error, low values (< 0.25) might be observed when high reproducibility is attained, and thus the design is considered as acceptable in these particular cases as well (19, 28). Furthermore, factor-response interactions were also evaluated by the coefficient plot analysis, where model refinement was attained by removing non-significant model terms ($p > 0.05$).

Optimization. – After model fitting and refinement, an optimizer run was carried out based on the parameter settings provided by the software. The optimizer run was injected five times and the obtained results were compared with the predicted results by the computational program using the prediction spreadsheet option.

Preliminary validation of the method after in silico *optimization*

Linearity. – Method linearity was tested in the range of 1–15 μg mL^{-1} through six sample points 1.0, 2.5, 5.0, 7.5, 10.0, 15.0 μg mL^{-1}. Table samples were prepared by dilution from a stock solution of 20 μg mL^{-1}. The determination was repeated five times for each concentration level.

Model selectivity. – Selectivity studies were conducted by recording the chromatograms of individual excipient samples, placebo formulation and placebo spiked with FAM prepared under the same conditions.

Model accuracy. – Method recovery was evaluated at three points (50, 100 and 150 %) of the target concentration. Individual samples were prepared with FAM-spiked placebo and repeated three times for each determination.

Robustness. – Method robustness was tested by applying a separate experimental design (Plackett-Burman model, fitted with the multiple linear regression method) (19). The model offers the possibility to assess the linear factor-response relationship, but no factor interactions can be evaluated by using the disclosed experimental model. In the case of robustness testing using experimental designs, four general outcomes (limiting cases) of the obtained results are defined. Amongst the various outcomes of the fitted and refined Plackett-Burman model, the limiting case of inside specification limits, but non-significant relationship is considered as an optimal result for robustness testing. For the robustness testing the same factors were defined as described in the previous experimental design with the following settings: flow rate 0.95–1.05 mL min^{-1}, column temperature 37.5–41.5 °C and the proportion of the phosphate buffer, pH = 3.0, between 28 and 32 (%, *V*/*V*). The experimental design returned 11 experimental runs.

Furthermore, method robustness was tested for individual changes in detection wavelength at 260 and 264 nm.

Model precision. – Placebo samples spiked with 5 mg of FAM were prepared on the same day by the same analyst and on two different days by two analysts. The analysis was carried out on six replicate samples for each determination. Instrument precision was tested on five replicate injections of the same sample.

*Limit of detection (*LOD*) and limit of quantification (*LOQ*).* – *LOD* and *LOQ* values were assessed at a 3:1 and 10:1 signal-to-noise ratio for six and three replicate determinations, resp.

Assay from the pharmaceutical dosage form

Ten tablets of Fampyra 10 mg prolonged-release tablets were pulverized and 200 mg of sample, corresponding to 5 mg FAM was admixtured with 500 mL phosphate buffer, pH = 6.8, sonicated in an ultrasound bath for 15 min and completed with the same solvent to 1 L in a volumetric flask. The final sample was filtered through a 0.45-μm Chromafil® Xtra PA-45/25 filter discarding the first 5 mL of filtrate. Active substance content was analysed from three individual samples.

Statistical analysis

Statistical analyses were carried out using the Minitab 17 software (Minitab Inc., State College, PA, USA) and Statistica 8.0 software (StatSoft, Tulsa, OK, USA).

Possible aberrant values in experimental design were detected and excluded using the Grubb's test for outliers at $\alpha = 0.05$ significance level ($p < 0.05$).

Shapiro-Wilk's test was used for the assessment of the normal distribution of the residuals, at a 95 % confidence interval. The null hypothesis, that the residuals do not follow a normal distribution, is rejected if *W* for FAM is greater than the critical tabulated value for the number of observations at $\alpha = 0.05$ and simultaneously $p > 0.05$. Furthermore, the plot of response values *vs.* corresponding concentrations was calculated with an acceptance limit of 2.5 % in comparison to target level response factor in the range of 50–150 % and 5.0 % in the range of tested linearity. ANOVA *F*-test and its test for lack-of-fit were used for the significance testing of the calibration curve (CI = 95 %). In order to assess the capability of the process to comply within specification limits, process capability (C_{pk}) was calculated for the plot of response values *vs.* corresponding concentrations at ± 10 % specification limits (acceptance criteria: $C_{pk} > 1.33$).

In order to statistically assess the variability of the sets of the results obtained for intra- and inter-day model precision, Student's *t*-test was used by setting the significance level at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Screening experimental design

Experiments no. 14 and 15 were excluded from DoE analysis due to inappropriate chromatographic results. Grubb's test performed after the exclusion of the aforementioned experiments did not result in the detection of outlier values. The experimental results are presented in Table II.

Table II. Experimental runs of the screening optimization DoE – Factor settings and response result set

Experiment No.	Run order	Flow rate (mL min^{-1})	Column temperature (°C)	Phosphate buffer proportion (%)	Tailing factor	Theoretical plate number (N)[b]	Retention time (min)
1	26	0.50	20	30	1.96	4423.7	5.91
2	5	1.00	20	30	1.80	3378.3	2.94
3	4	1.25	20	30	1.71	3262.0	2.35
4	27	0.50	30	30	1.94	5152.0	5.91
5	1	1.00	30	30	1.71	4124.0	2.95
6	20	1.25	30	30	1.62	4101.7	2.37
7	8	0.50	40	30	1.92	5308.7	5.81
8	16	1.00	40	30	1.65	4580.0	2.91
9	15	1.25	40	30	1.67	4647.0	2.33
10	3	0.50	20	50	1.90	4127.0	5.09
11	2	1.00	20	50	1.52	1771.7	2.54
12	18	1.25	20	50	1.45	1393.3	2.01
13	17	0.50	30	50	2.02	5163.7	4.98
14[a]	21	1.00	30	50	–	–	–
15[a]	14	1.25	30	50	–	–	–
16	24	0.50	40	50	1.91	6013.0	4.94
17	28	1.00	40	50	1.65	2899.3	2.47
18	19	1.25	40	50	1.84	2414.3	1.96
19	12	0.50	20	70	2.21	5373.3	4.98
20	9	1.00	20	70	1.87	2423.7	2.48
21	13	1.25	20	70	1.83	1976.7	1.95
22	23	0.50	30	70	2.23	6491.7	4.85
23	10	1.00	30	70	1.92	3572.7	2.41

Experiment No.	Run order	Flow rate (mL min^{-1})	Column temperature (°C)	Phosphate buffer proportion (%)	Tailing factor	Theoretical plate number (N)[b]	Retention time (min)
24	30	1.25	30	70	1.79	3266.7	1.92
25	22	0.50	40	70	2.43	6882.3	4.80
26	25	1.00	40	70	1.72	4465.0	2.38
27	29	1.25	40	70	1.69	3731.0	1.89
28	7	1.00	30	50	1.79	2544.3	2.50
29	6	1.00	30	50	1.92	2173.7	2.48
30	11	1.00	30	50	1.97	2392.7	2.49

[a] Excluded experiments due to inappropriate chromatograms.
[b] Defined per column length.

Table III. Settings of the optimized chromatographic conditions – comparison of software predicted vs. *experimentally observed results of the developed method*

Optimized chromatographic conditions according to experimental design software					
Flow rate (mL min^{-1})		PB proportion in mobile phase (%)[a]			Column temperature (°C)
1.0		30.0			39.5
Predicted *vs.* observed results					
	Min	Predicted	Max	Observed	Difference (predicted *vs.* observed)
Retention time (min)	2.78	2.91	3.05	2.88	–0.03
Theoretical plate number (N)[b]	4077.4	4445.0	4812.6	4771.6	+326.6
Tailing factor	1.62	1.70	1.78	1.69	–0.01

[a] PB – phosphate buffer, pH = 3.0.
[b] Defined per column length.

Significant regression models ($p < 0.05$) were obtained in the case of all three studied responses (retention time, theoretical plate number and tailing factor); lack-of-fit was only observed in the case of retention time ($p = 0.003$). In the latter case, low model validity is explained by the high reproducibility (0.99995) of the replicate runs. The identical results obtained for retention time under the replicate runs, as desired in HPLC method development, leads to a low pure error inside the model, or namely high reproducibility. Usually, in this case the model error is substantially greater than the pure error, resulting in a higher lack-of-fit, which in turn persuades in low model validity. The analysis of the model returned good model performance indicator values (Fig. 3a), as R^2 and Q^2 values are situated between 0.63–0.98 and 0.61–0.97, resp. The difference between the values of goodness of fit (R^2) and goodness of predictability (Q^2) does not exceed the requirement of 0.2–0.3. The model validity for tailing factor and theoretical plate number is above the minimum threshold of 0.25, with values of 0.75 and 0.61, resp. The reproducibility values of the responses are all superior to the minimal criteria of 0.5, ranging from 0.80 to 0.99.

The analysis of the coefficient plots (Fig. 3b) revealed that retention time is strongly influenced by the flow rate and phosphate buffer proportion and shows a negative correlation with the flow rate and the proportion of the buffer in the mobile phase. Similarly, in the case of the tailing factor, the flow rate has a negative impact on response results, whilst in terms of buffer concentration a positive correlation was found. The theoretical plate number is influenced in an identical manner by the flow rate as observed in the previous cases. Contrarily to these, in the case of theoretical plate number a strong influence by column temperature can be observed, whilst the effect of the phosphate buffer portion is negligible. Although indicating a curved surface inside the model, the detected factor interactions (flow × PB) and quadratic terms (flow × flow, PB × PB) for retention time and theoretical plate number were retained, as deletion of these particular influencing factors would result in performance model indicator deterioration.

Optimizer run. – Based on the fitted and refined experimental design, a placebo sample spiked with 5 mg FAM was injected five times using the factor settings offered by the software and the results were compared with those predicted by the computational design. The optimized factor settings and comparison of the predicted *vs.* observed result sets are presented in Table III.

Under the set of experimental conditions (flow rate 1.0 mL min^{-1}, column temperature 39.5 °C and 30 % PB) the prediction of the program proved to be reliable, as a sample retention time of 2.88 min is marginally inferior to the foreseen 2.91 min and a peak tailing of 1.69 is properly close to the predicted 1.70. Regarding the values of the theoretical plate number, a higher value was observed in comparison to the predicted one, but still lying within the confidence interval provided by the software. A typical chromatogram under the optimized chromatographic conditions is presented in Fig. 2b.

Model validation results

Selectivity. – Under proposed settings, FAM can be identified as a sharp, well-defined peak 262 nm, from both the standard solution and pharmaceutical dosage form, in comparison to placebo formulation (see Figs. 2a-c); no interference of the excipients can be observed (see Fig. 2a).

Linearity. – The method proved to be linear in the tested range of 1–15 μg mL^{-1}, with a coefficient of determination of $R^2 = 0.9996$. The Shapiro-Wilk's test for the normal distribu-

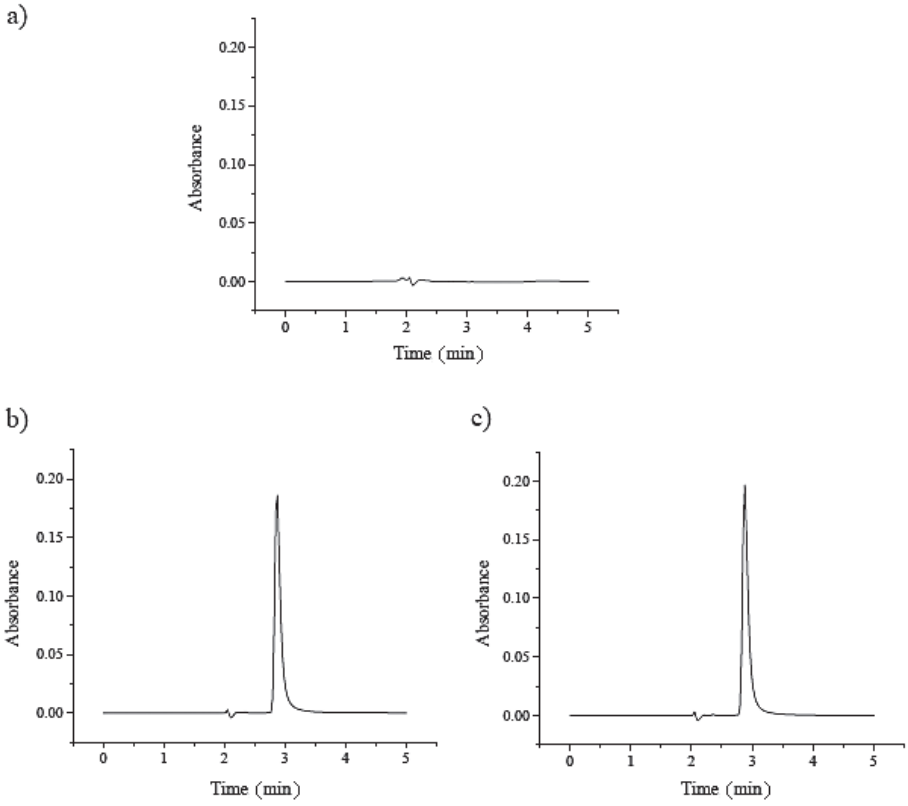

Fig. 2. a) Chromatogram of placebo sample, b) typical chromatogram of placebo spiked with fampridine (5 μg mL^{-1}), c) chromatogram of a real sample obtained from the original commercial product (5 μg mL^{-1} FAM).

tion of the residuals was passed, as the obtained W_{FAM} = 0.866 was greater than the critical threshold of $W_{CRIT.}$ = 0.788 (n = 6, α = 95 %), p = 0.212. The ANOVA F-test revealed a significant relationship between the predictors and responses ($p < 0.05$) and no lack-of-fit was observed ($p > 0.05$). The capability analysis of the plot of response values *vs.* concentration resulted in a C_{pk} = 1.72, indicating that the model is well controlled within the proposed linearity range. Statistical results are given in Table IV.

Robustness. – The method proved to be robust in the tested range as minor changes in the chromatographic conditions did not influence the detectability of the analyte (Table V). This is also supported by the results of the Plackett-Burman experimental design, where no significant relationship was found between the factors and responses in the case of tailing factor and theoretical plate number ($R^2 < 0.5$). Concurrently, the low goodness of predictability values indicates that the model could not detect remarkable modifications in response values that are due to minor changes in factor settings.

Table IV. Model analytical merits of the developed HPLC method

Parameter	Result	Statistical result
Linearity (μg mL^{-1})	1–15 μg mL^{-1}	R^2 = 0.9996 W_{FAM} = 0.866[a] (p = 0.212) F = 22,132.69 (p < 0.05)[b] F = 2.35 (p > 0.05)[c] C_{pk} = 1.72[d]
Accuracy (%)	98.7 ± 1.9	R^2 = 0.9998
Intraday precision (RSD, %)[e,f]	0.4–0.7	$t_{<analyst\ 1\ day\ 1\ vs.\ analyst\ 2\ day\ 1>}$ = 1.548 p = 0.153
Inter-day precision (RSD, %)[e,f]	0.5	$t_{<analyst\ 1\ day\ 1\ vs.\ analyst\ 1\ day\ 2>}$ = 1.682 p = 0.123 $t_{<analyst\ 2\ day\ 1\ vs.\ analyst\ day\ 2>}$ = 0.237 p = 0.816
Instrument precision (RSD, %)[g]	0.5	–
LOD (μg mL^{-1})	0.24	–
LOQ (μg mL^{-1})	0.78	–
Assay from tablets (%)[h,i]	100.3	–

[a] $W_{CRIT.}$ = 0.788 – critical tabulated value of Shapiro-Wilk's test.
[b] Critical tabulated value of ANOVA *F*-test, F = 4.20.
[c] Critical tabulated value of ANOVA *F*-test for lack-of-fit, F = 2.70.
[d] Requirement, C_{pk} > 1.33.
[e] Critical value of t = 2.228, df = 10.
[f] n = 6, [g] n = 5, [h] n = 3.
[i] Compliance with the label claim.

In contrast to the screening experimental design, in the case of the Plackett-Burman model higher model validity values are attained (Fig. 3c), whereas in the case of retention time a negative to positive switch is observed, indicating that the model is valid in the proposed testing range.

The analysis of the coefficient plots (Fig. 3d) shows that the theoretical plate number and tailing factor are not influenced in a significant manner by any of the defined factors. In contrast, retention time is strongly influenced by the flow rate and the proportion of the phosphate buffer in the mobile phase, both having a negative correlation with the mentioned response. These tendencies are similar to those observed in the screening experimental model. The existence of strong factor to response correlations explains the higher R^2 and Q^2 values, since, as expected, minor changes in flow rate and mobile phase composition have a slight effect on the retention of the active substance to the column's stationary phase, thus modifying retention time in a systematic manner.

Negligible changes in detection wavelength (± 2 nm) did not influence the selected responses in a critical manner, resulting in a tailing factor of 1.66 and a theoretical plate number of 4712 in both cases, and a recovery of 99.5 and 99.9 % at 260 and 264 nm, resp.

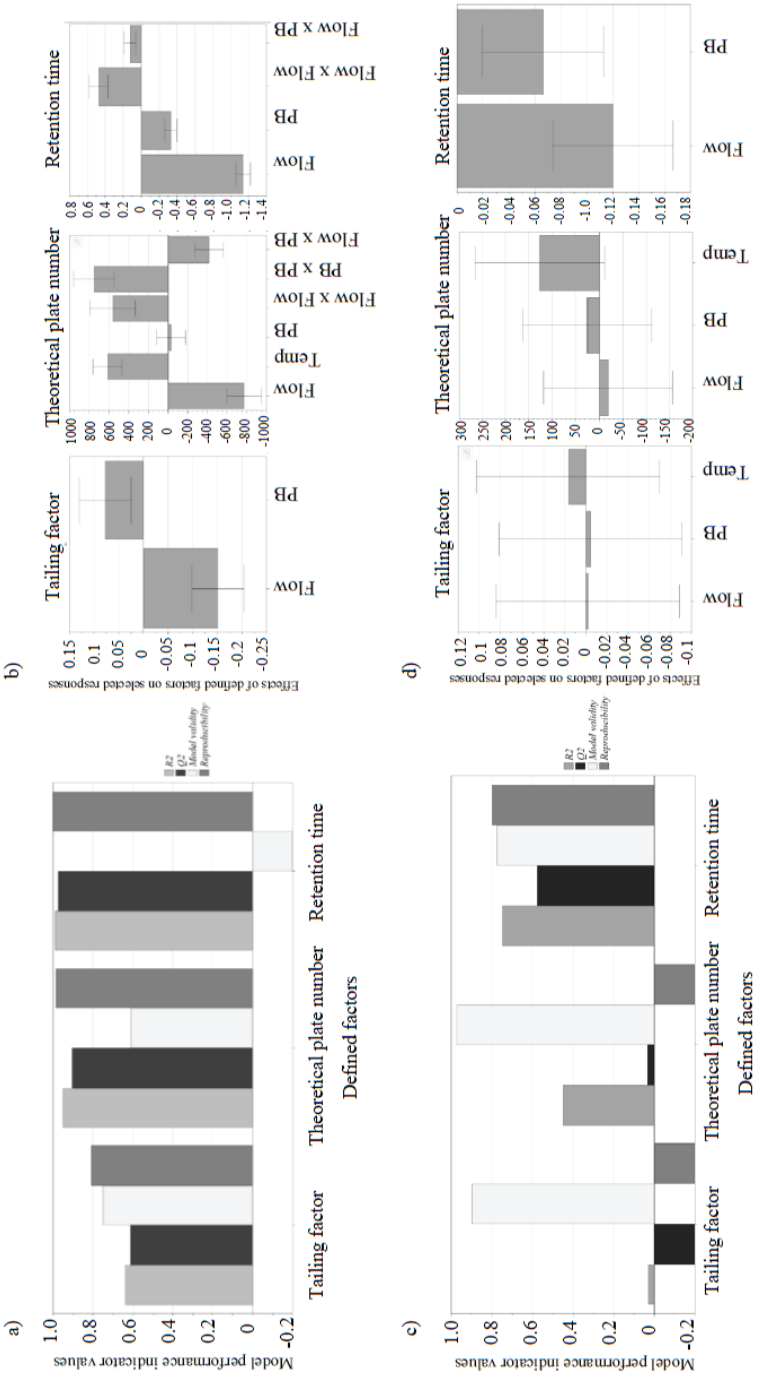

Fig. 3. Summary of: a) fit plot and b) coefficient plot of screening experimental design. Summary of: c) fit plot and d) coefficient plot of Plackett-Burman design.

Accuracy. – The mean recovery of the method was 98.7 %, value lying in-between 95.8 and 101.8 % for the 50–150 % interval (Tables IV and VI). Also, the linearity of the spiked samples shows a good linear correlation, with $R^2 = 0.9998$.

Precision. – The performed Student's *t*-test revealed that there is no statistical difference between the sample set prepared by the same analyst on the same day (intra-day precision) and between the two analyst's sample set prepared on two different days (inter-day precision). The obtained results showed low variability between the samples, with an RSD of 0.6 % (100.5–103.3 %). An instrument precision with an RSD of 0.5 % (99.9–101.3 %) was obtained after five successive injections of the same sample solution (Tables IV and VI).

*Limit of detection (*LOD*) and limit of quantification (*LOQ*).* – The limits of detection and quantification of the developed method are 0.24 and 0.78 μg mL^{-1}, resp.

Assay of the pharmaceutical dosage form

Three replicate samples yielded an average recovery of 100.6 % (RSD = 0.7 %) from FAM tablet formulation (Tables IV and VI) (Fig. 2c).

Method benefits

The proposed analytical method offers a high throughput analysis for FAM and was aimed to be useful in the assay of the active pharmaceutical ingredient from tablets and

Table V. Experimental runs of the robustness testing DoE – Factor settings and response result set

Experiment no.	Flow rate (mL min^{-1})	Column temperature (°C)	Phosphate buffer proportion (%)	Retention time (min)	Tailing factor	Theoretical plate number (N)[a]
1	1.05	28	37.5	2.85	1.81	4847.0
2	1.05	32	37.5	2.85	1.81	4847.0
3	1.05	32	41.5	2.72	1.90	5003.0
4	0.95	32	41.5	3.01	1.79	5247.0
5	1.05	28	41.5	2.92	1.80	5078.0
6	0.95	32	37.5	2.97	1.81	4857.0
7	0.95	28	41.5	3.18	1.90	5032.5
8	0.95	28	37.5	3.14	1.84	4792.5
9	1.00	30	39.5	2.87	1.71	4991.0
10	1.00	30	39.5	2.88	1.59	4772.0
11	1.000	30	39.5	2.91	1.82	4576.0
Average				2.93	1.79	4913.0
Difference to predicted optimizer results				+0.02	+0.09	+468.0
Difference to observed optimizer results				+0.06	+0.10	+141.4

[a] Defined per column length.

Table VI. Results obtained for model studies of accuracy, precision and pharmaceutical dosage form

Model accuracy study			
FAM in the real blank			
Theoretical FAM in the real blank (μg mL^{-1})	Average FAM recovered (μg mL^{-1})[a]	Average recovery (%)[a]	RSD (%)[a]
2.500	2.503	100.1 ± 1.3	1.3
5.000	4.948	99.0 ± 2.2	2.2
7.500	7.273	97.1 ± 0.8	0.9
	Average[b]	98.7 ± 1.9	2.0

Model precision study					
Theoretical FAM in sample (μg mL^{-1})	Sample no.	Inter-day precision		Intra-day precision	Average (%)
		Concentration found (%)			
		Analyst 1	Analyst 2	Analyst 2	
5.000	1	101.8	102.6	102.5	102.1
	2	102.0	101.8	103.0	
	3	100.5	101.3	102.1	
	4	102.1	102.8	101.9	
	5	102.4	103.3	101.9	
	6	101.5	102.2	102.0	
	Average	101.7	102.3	102.2	
	RSD (%)	0.6	0.7	0.4	0.6

Assay of pharmaceutical dosage form		
Expected FAM (μg mL^{-1})[a]	Sample no.	FAM found (%)
5.000	1	100.4
	2	100.9
	3	99.5
	Average	100.3
	RSD (%)	0.7

[a] n = 9, [b] n = 27, [c] Based upon label claim.

dissolution profile samples. When compared to the already available methods (see Table VII) that aimed only the assay of the active substance (12), the novel method has a lower linearity range of 1–15 μg mL^{-1} *vs.* 20–80 μg mL^{-1}. Comparing the other analytical validation parameters, the novel method surpasses the currently accessible method (12), espe-

Table VII. Comparison of the proposed method to the already available HPLC methods

Stationary phase	Mobile phase	Retention time (min)	Detection (λ)	Analytical merits	Aim	Reference
C18 (100 mm × 4.6 mm, 3.5 μm)	Gradient elution: A – sodium-1-heptane sulfonate, monobasic potassium phosphate and phosphoric acid in water; B – acetonitrile	–	275 nm	–	–	17
Inertsil ODS 3V C18 (150 mm × 4.6 mm, 5 μm)	Gradient elution: phosphate buffer (pH = 4) with 1-octane sulphonic acid and CAN	14.02	PDA (260 nm)	Linearity: not indicated *LOD* = 0.080 μg mL^{-1} *LOQ* = 0.280 μg mL^{-1}	Determination of related substances in FAM drug substance and tablet dosage forms	11
Inertsil C18 (150 mm × 4.6 mm, 5 μm)	Isocratic elution: phosphate buffer (pH = 4):ACN (30:70)	2.43	UV (298 nm)	Linearity: 20-80 μg mL^{-1} *LOD* = 0.710 μg mL^{-1} *LOQ* = 2.160 μg mL^{-1}	Determination of dalfampridine in bulk and formulation	12
XBridge C18 (150 mm × 4.6 mm, 5 μm)	Gradient elution: ammonium formate (pH = 9) and ACN	4.19	PDA (220 nm)	Linearity = 1-500 μg mL^{-1} *LOD* = 0.100 μg mL^{-1} *LOQ* = 1.000 μg mL^{-1}	Simultaneous determination of teriflunomide, dimethyl fumarate and FAM in human plasma	16
Xterra-RP18 (250 mm × 4.6 mm, 5 μm)	Gradient elution: 1-octanesulphonic acid sodium salt monohydrate, ammonium acetate and triethylamine (pH = 4) and MeOH	11.97	UV (240 and 282 nm)	Linearity: 0.225–1.687 μg mL^{-1} *LOD* = 0.075 μg mL^{-1} *LOQ* = 0.225 μg mL^{-1}	Impurities in FAM API	10
Zorbax silica HILIC (250 mm × 4.6 mm, 5 μm)	Isocratic elution: ammonium formate (pH = 5):MeOH:ACN (15:8.5:76.5)	50.21	PDA (280 nm)	Linearity range, *LOD* and *LOQ* not indicated	Impurities in FAM drug substance	13
LiChrosphere 60 RP-select B (250 mm × 4.6 mm, 5 μm)	Isocratic elution: phosphate buffer (pH = 3): MeOH:ACN (30:5:65)	2.88	DAD (262 nm)	Linearity: 1-15 μg mL^{-1} *LOD* = 0.240 μg mL^{-1} *LOQ* = 0.780 μg mL^{-1}	Determination of FAM in pharmaceutical dosage form	This paper

ACN – acetonitrile, API – active pharmaceutical ingredient, FAM – famotidine, MeOH – methanol

cially in terms of *LOD* and *LOQ* of 0.24 μg mL^{-1} *vs.* 0.71 μg mL^{-1} and 0.78 μg mL^{-1} *vs.* 2.16 μg mL^{-1}, resp. Furthermore, in terms of retention time, the proposed method performs better or equivalently in contrast to other analytical reports. Taking into consideration the FDA requirement (29) for the volume of dissolution media (900 mL) and the amount of the active substance (10 mg), a general concentration span between 1.11 μg mL^{-1} (10 % of active substance dissolved) and 11.11 μg mL^{-1} (100 % of active substance dissolved) would be obtained at specified sampling times (0.5–12 h). As method validation was carried out nearby the aforementioned concentration range, the novel method might perform worthier when compared with other methods and is applicable without time-consuming sample preparation. Furthermore, the developed method might be considered as a surrogate for the available compendial method, having its benefit in the simpler and more available mobile phase components.

CONCLUSIONS

The developed method offers high throughput, cost-effective sample analysis from both bulk samples and pharmaceutical dosage form. The applied computational method development offers the possibility to collect *in silico* information regarding the chromatographic particularities following modifications in factor settings. The newly proposed method for the assay of fampridine was preliminarily validated as per the currently available international guidelines and fulfilled the model validation requirements for linearity, accuracy, robustness and precision. Furthermore, the development and validation of the present method employing Quality by Design approach support the essentiality of software-controlled methodology in modern pharmaceutical research and development.

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