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Robustness testing of a liquid chromatography method for the determination of vorozole and its related compounds in oral tablets

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Abstract

The robustness of a method for the determination of vorozole in oral tablets was examined by applying a two-level, seven factor Plackett-Burman statistical experimental design. Five method variables that are sensitive to variation, especially during method transfer, were evaluated for their influence on the system suitability criteria set in the method procedure and on the analysis time. The method variables were investigated in a specified range above and below the nominal method conditions. They included the concentration of an ion-pairing agent, the percentage organic modifier at the start of the linear gradient, the mobile phase flow rate, the percentage organic modifier at the end of the linear gradient and the pH of the mobile phase. Two dummy factors were included in the design to estimate the experimental error. It was found that none of the five studied variables affected significantly (t-test, $\alpha = 0.01$) the capacity factor, the tailing factor or the analysis time. The resolution of the critical peak pair on the other hand, was significantly influenced by the factor pH. However, the responses for the resolution of all the experimental runs in the design were well above the system suitability limit stated in the normal assay procedure. Therefore, the method can overall be considered robust.

Keywords: Vorozole; Robustness testing; Plackett-Burman experimental design; Pareto plots

1. Introduction

According to the ICH [1] and the USP [2], robustness is the capability of an analytical procedure to remain unaffected by small but deliberate variations in the method parameters. Robustness testing identifies variables that have a significant effect on the outcome of a method. It allows setting limits for the method variables and therefore provides useful information, especially for method transfer. The variations in the method parameters are selected in such a way that it represents the variation which is expected to occur if the method is performed under different conditions.

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In robustness testing statistical experimental designs are applied in order to investigate a large number of variables with less experimental effort and in a short time [3]. For this purpose one applies screening designs such as Plackett-Burman and fractional factorial designs [4–6]. Robustness testing is considered as a part of method validation [1,2,6] and it is usually performed at the end of the method validation process, prior to method transfer. Nowadays it is recommended to perform robustness evaluations much earlier in the life cycle of a method [1,7]. In this way possible method problems can be optimised before starting full validation, which is usually the most time consuming process during the life cycle of a method.

In the present study the robustness of a method for the determination of vorozole in 2.5 mg oral tablets was examined by applying a two-level, seven factor Plackett-Burman statistical experimental design. Vorozole is a anti tumour agent which was developed at Janssen Research Foundation and has been reported to be very promising in the treatment of breast cancer [8,9]. The analytical method is a gradient liquid chromatography (LC) method performed under reversed phase conditions. By this method it is possible to separate and determine vorozole and its potential impurities A, B and C in one run. The structure of this compound is shown in Fig. 1. Five method variables that are sensitive for variation, especially during method transfer, were evaluated for their influence on the system suitability test (SST) criteria set in the method procedure and on the analysis time (retention time of the last peak). The SST criteria that were used as responses were the resolution of the first peak pair, the capacity factor and the tailing factor of the main peak. The method variables were investigated in a specified range above and below the nominal method conditions. They included the concentration of the ion-pairing agent tetrabutylammonium hydrogensulphate (TBA) (range: 9.5–10.5 mM), the percentage organic modifier at the start of the linear gradient (range: 19–21%), the mobile phase flow rate (range: 1.9–2.1 ml min^{-1}), the percentage organic modifier at the end of the linear gradient (range: 48–52%) and the pH of the TBA solution (range: 2.1–2.3). Two dummy factors were included in order to estimate the experimental error.

2. Theory

The selected experimental design was a two-level, seven factor Plackett-Burman design that is usually used for screening purposes. The design requires performing eight experiments with different level combinations. The influence of the five method variables on the resolution of the first peak pair, the capacity factor, the tailing factor and the analysis time was examined by the experimental design and is expressed as main effects of the factors. Resolution, capacity and tailing factor are calculated according to USP guidelines [2]. The main effects of the factors are calculated according to generally applied formulas [4]. Two dummy factors were included in the design for the estimation of the experimental error. A dummy factor is an imaginary variable of which the change from one level to the other does not represent a physical change [4]. They are used to obtain two degrees of freedom in the statistical analysis. Although this number is rather small, it is still acceptable in this case because the statisti-

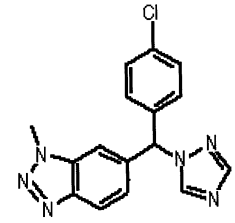

Fig. 1. Vorozole.

Table 1
Solvent gradient

Time (min)	0	10	11	17
% A[a]	80	50	80	80
% B[b]	20	50	20	20

[a] A: 0.01 M tetrabutylammonium hydrogen sulphate (TBA) in water. Adjust to the required pH with NaOH 0.1M or HCl 0.1M.

[b] B: acetonitrile.

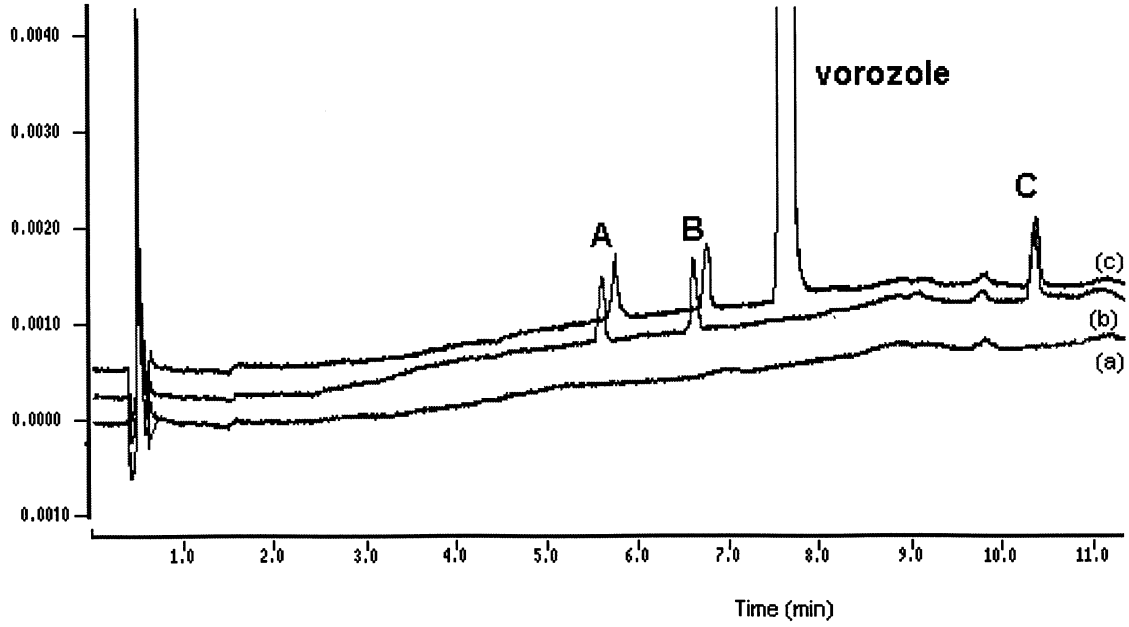

Fig. 2. Chromatograms obtained at nominal conditions: (a) a blank solution; (b) a solution containing 0.5% related compounds and (c) a chromatogram of the test solution containing 100% vorozole and 0.5% related compounds.

cal test will not be very sensitive. This is suitable for applications where the experimental error is quite small, a situation that is true for the latest HPLC instruments. With a very sensitive test a small drift is easily detected as being statistically significant. Something that should be avoided from the practical point of view. The significance of the factor effects is determined according to a t-test. First a t-value (Eq. (1)) is calculated which is compared with a tabulated two-sided critical t-value at a significance level (α) and n_{dummy} degrees of freedom.

$$t - \text{value} = \frac{|E_x|}{\text{SE}} \geq t_{\text{critical}} \tag{1}$$

and

$$\text{SE} = \sqrt{\frac{\sum E_i^2 \text{dummy}}{n_{\text{dummy}}}} \tag{2}$$

where E_x and E_{dummy} are the calculated effects of a factor and a dummy, respectively. SE is the standard error of E_x and n_{dummy} is the number of dummy factors included in the design.

The calculated t-values (standardized effects) are plotted from large to small values in Pareto plots. The critical t-value is then used to make a visual decision of the significance of the effects. It is also possible to construct a confidence interval (CI) for each effect:

$$\text{CI} = E_x \pm t_{(n-\text{dummy},\,\alpha)} * \text{SE} \tag{3}$$

3. Experimental

3.1. Chemicals

The related substances A, B and C and vorozole reference standard were obtained from Janssen Research Foundation (Beerse, Belgium). Methanol was purchased from J.T. Baker (Deventer, The Netherlands), acetonitrile HPLC-grade from Acros (Geel, Belgium) and tetrabutylammonium hydrogen sulphate was obtained from Fluka (Buchs, Zwitserland). Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were obtained from Merck (Darmstadt, Germany).

Table 2
The selected method factors with their level ranges

Factor	Level		
	−1 = low	1 = high	0 = nominal
Dummy 1	−1	1	0
Concentration of TBA	9.5	10.5	10 mM
% Acetonitrile at the start of the gradient	19	21	20%
Flow of the mobile phase	1.9	2.1	2.0 ml min^{-1}
% Acetonitrile at the end of the gradient	48	52	50%
pH of the TBA solution	2.1	2.3	2.2
Dummy 2	−1	1	0

Table 3
The investigated responses and their experimentally obtained results in the design

[SST-limit[a] experiment]	Resolution[b] Min. 6.9	Capacity factor[c] Min. 9.6	Tailing factor[c] Max. 1.3	Analysis time[d] –
1	10.0	15.36	1.09	10.93
2	8.3	14.03	1.05	10.24
3	10.0	13.90	1.10	10.02
4	8.0	13.60	1.07	10.31
5	10.5	13.52	1.03	10.16
6	10.0	12.14	1.04	9.17
7	8.1	13.49	1.06	9.68
8	8.0	13.35	1.09	9.80

[a] The SST-limit is the minimal or maximal value that is allowed for results to be acceptable.
[b] Resolution between A and B.
[c] Capacity and tailing factor of the main (vorozole) peak.
[d] Retention time of the last peak: C. SST: system suitability test.

3.2. Method description

3.2.1. Solutions

Standard solution related compounds: weigh approximately 5 mg of related compounds A, B and C into a 200 ml volumetric flask. Dissolve in and dilute to volume with methanol/acetonitrile (1/1). Standard solution: Accurately weigh approximately 50 mg vorozole reference standard into a 100 ml volumetric flask. Dissolve in and dilute to volume with methanol/acetonitrile (1/1). Test solution: transfer 10 ml of standard solution and 1 ml of standard solution related compounds into a 50 ml volumetric flask. Dilute to volume with methanol/acetonitrile (1/1). Aqueous phase of the mobile phase: 0.01 M tetrabutylammonium hydrogen sulphate (TBA) in Milli-Q (Millipore, Milford, USA) water. Adjust to the required pH with NaOH 0.1M or HCl 0.1M.

3.2.2. Chromatographic conditions

The separation was performed on a 10 cm × 4.6 mm ID column, packed with 3 μm particle size Hypersil BDS-C_{18} phase, at ambient temperature on a Waters Alliance 2690 separation module. Methanol was used as autosampler flush solvent. Detection was performed by a Waters 486 UV detector set at 230 nm. Gradient elution was performed with a solvent gradient as described in Table 1, at a flow rate of 2.0 ml min^{-1}.

3.3. Software

The statistical analysis of the data was performed by the aid of the software package: STATGRAPHICS-PLUS (2.1) for Windows, (Manugistics, Rockville).

Table 4
The calculated main effects of the factors and their 99% confidence intervals

Factor	Responses							
	Resolution		Capacity factor		Tailing factor		Analysis time	
	Effect	CI[a] ($\alpha = 0.01$)	Effect	CI ($\alpha = 0.01$)	Effect	CI ($\alpha = 0.01$)	Effect	CI ($\alpha = 0.01$)
Concentration of TBA	−0.1	±0.785	−0.083	±5.119	−0.008	±0.055	+0.089	±1.858
% Acetonitrile at the start of the gradient	−0.2	±0.785	−1.048	±5.119	−0.012	±0.055	−0.413	±1.858
Flow rate of the mobile phase	+0.2	±0.785	−0.733	±5.119	−0.043	±0.055	−0.396	±1.858
% Acetonitrile at the end of the gradient	−0.05	±0.785	−0.898	±5.119	+0.013	±0.055	−0.785	±1.858
pH of the TBA solution	**−1.95**	**±0.785**	−0.138	±5.119	+0.008	±0.055	−0.084	±1.858
Dummy 1	+0.05		−0.676		−0.008		−0.249	
Dummy 2	+0.1		+0.283		+0.003		+0.09	

[a] CI, confidence interval range: this value is added to or subtracted from the effect value to obtain the upper and lower confidence interval levels.

4. Results and discussion

Chromatograms indicating the specificity of the method are shown in Fig. 2. These chromatograms are obtained with the chromatographic conditions as described in the normal assay procedure. Fig. 2a is a typical blank chromatogram. Fig. 2b is a chromatogram of a mixture corresponding to 0.5% of related compounds with regard to the nominal concentration (100%) of vorozole. Fig. 2c is a chromatogram of a mixture corresponding to 100% of vorozole and 0.5% of related compounds. As can be observed all the related compounds are nicely separated from each other and from the main peak of vorozole.

The selected factors and their level ranges are summarised in Table 2. For the concentration of TBA (range: 9.5–10.5 mM) and the percentage acetonitrile at the start (range: 19–21%) and the end (range: 48–52%) of the gradient a level range of 5% above and below the nominal method conditions was chosen as extremes. Selecting the extreme levels as a percentage of the nominal level is not always appropriate, however, in this case this is not considered as a problem. From the equipment qualification tests of the used HPLC system it is known that an error of ± 3% on the slope of the gradient is tolerated over the whole range. For the pH (range: 2.1–2.3) and the mobile phase flow rate (range: 1.9–2.1 ml min^{-1}) a level range of 0.1 absolute units above and below the nominal conditions was chosen as extremes.

The investigated responses together with their experimentally obtained values for the experiments in the design are summarised in Table 3. The system suitability limits are also reported. The separation between the related compounds A and B is a part of the system suitability test. According to the normal assay procedure a minimum resolution of 6.9 is required for this peak pair. This has to be verified each time the method is applied in order to check the suitability of the system. For this reason it is important to know the influence of the method variables on this parameter. System suitability limits are also provided for the capacity and the tailing factor of the main (vorozole) peak. These two parameters are therefore also investigated as responses in this study. The analysis time is the fourth response

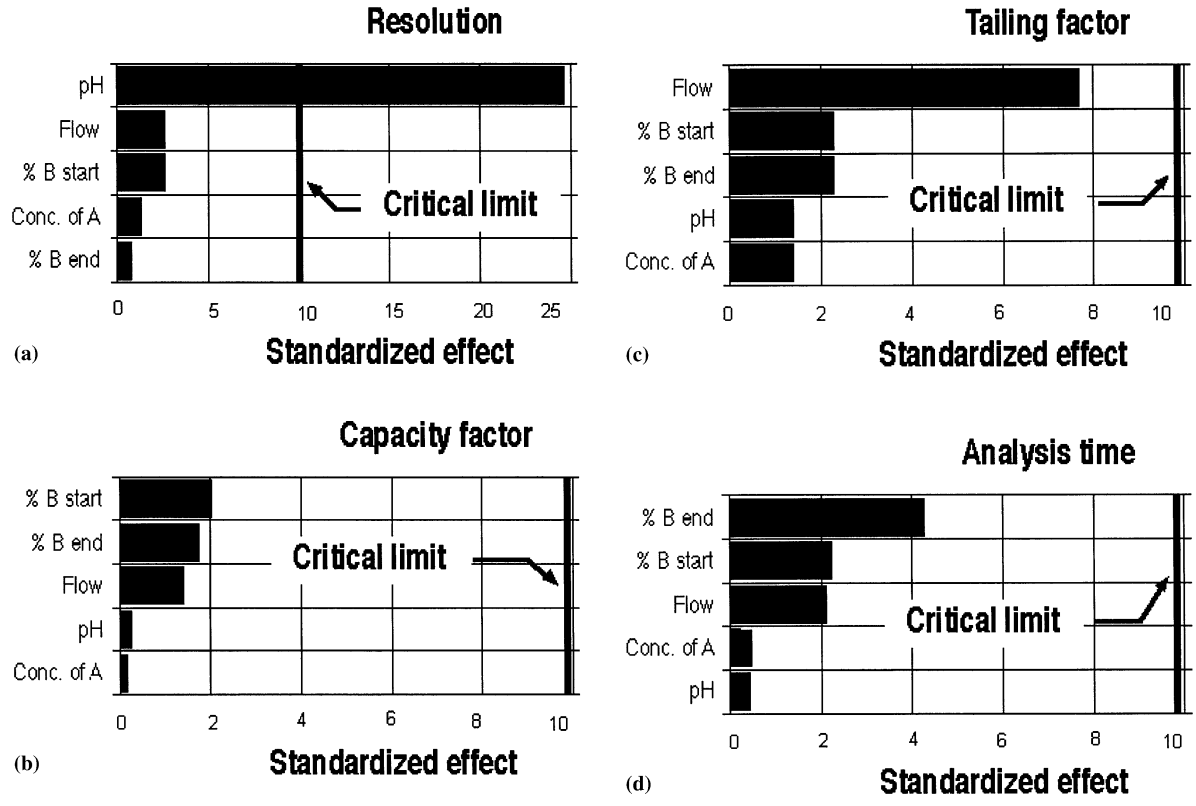

Fig. 3. Standardised Pareto charts for the responses (a) the resolution; (b) the capacity factor, (c) the tailing factor and (d) the analysis time.

that is included in this study. The time of analysis (retention time of the last peak) is important as it determines the run time.

As can be observed in Table 3, in none of the experiments proposed by the design a result is obtained that violates these limits. In the analysis of the data obtained after performing all the experiments proposed by the design, main effects were calculated for each factor. The effects were calculated with the aid of the software program: STATGRAPHICS-PLUS (2.1) for Windows. The calculated effects of each factor for all the responses are summarised in Table 4. A 99% confidence interval for the effects was also calculated. When zero is included in the confidence interval range, it is concluded that the effect of the factor is not significantly different from zero. This means that the effect is not significant and can be due to experimental error. It can be concluded from Table 4 that the pH of the TBA solution has a significant effect on the resolution.

Another way to demonstrate the statistical significance of the factors is by visual evaluation using standardised Pareto plots (Fig. 3). The calculated t-values (Eq. (1)) for the factors (or standardized effects) are plotted in the large to small order. In order to make a statistical decision, the critical t-value is also plotted perpendicular to that of the effects. An effect is considered significant when its standardised value exceeds the critical t-value. In Fig. 3a the results for the resolution response is presented. As can be noticed, the pH has a significant effect on the resolution, as was also derived from Table 4. Fig. 3b–d represent the results for the responses capacity factor, tailing factor and analysis time, respec-

tively. None of the factors result in significant effects. From these observations it is demonstrated that the method is robust towards all the factors, except for the pH. However, if one considers the system suitability limit and the results obtained in Table 3, then it can be observed that all the experiments of the design resulted in response values well above this limit. This demonstrates that a small deviation in pH does not imply practical relevance. However, care has to be taken during pH adjustment of the TBA solution, so that deviations of the prescribed pH are not excessive. Although none of the studied factors are critical for the analysis time, it is recommended to delay the gradient end time slightly. This would avoid the risk that compound C is eluting at a steep gradient condition and therefore increase the robustness of the method further.

5. Conclusions

From the results of this study it can be concluded that the method is robust for most of the factors that have been evaluated. The resolution can be influenced by the pH of the mobile phase. The statistically significant effect of the pH is not considered relevant in practice, provided that pH adjustments are performed with normal analytical care. Therefore, the method can be considered robust overall.

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