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Original research paper

# Use of chemometrics for development and validation of an RP-HPLC method for simultaneous determination of haloperidol and related compounds

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A rapid resolution reversed-phase high performance liquid chromatographic (RR RP-HPLC) method has been developed and validated for simultaneous determination of haloperidol and six related compounds. Investigated matrix was a laboratory mixture of a therapeutic active substance haloperidol and its six related compounds in concentration ratio 300:1. Experimental design was used during method optimization (full factorial $2^3$ design) and robustness testing (Central Composite Circumscribed design). Three factors: organic phase variation during gradient elution, flow rate and gradient rise time were independent variables. To estimate the system response during the optimization procedure and robustness testing, resolution ($R_s$) and a chromatographic response function (*CRF*) were used. Chromatography was performed with the mobile phase containing phosphate buffer pH 6.5 and acetonitrile as organic modifier. Separation was achieved using gradient elution (organic phase fraction changed linearly from 20 to 72 %) over 7 min. A Zorbax Eclipse XDB C18 Rapid Resolution HT 4.6 mm × 50 mm, 1.8 μm particle size, column was used at 25 °C at a flow rate of 1.5 mL $min^{-1}$. UV detection was performed at 230 nm. The total time for chromatographic separation was 5.5 min with a total analysis time of 7.0 min. The method was validated for its linearity, precision, modal recovery and robustness.

*Keywords*: haloperidol, impurities, RP-HPLC, validation, experimental design

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Haloperidol is one of the most widely used antipsychotic drugs in the treatment of schizophrenia, mania and other psychiatric disorders (1). Chemically, haloperidol (Hal) is 4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone. The drug has six known related compounds specified as impurities: 4-(4-chlorophenyl)-4-hydroxy

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piperidine (Imp. 1), 4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-hydroxyphenyl)--1-butanone (Imp. 2), 4-fluorobenzoic acid (Imp. 3), *trans*-4-[4-(4-chlorophenyl)-4-hydroxy--1-piperidinyl]-1-(4-fluorophenyl)-1-butanone, *N*-oxide monohydrate (Imp. 4), *cis*-4-[4-(4--chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone *N*-oxide (Imp. 5) and 4-[4-(4-chlorophenyl)-3,6-dihydro-1(2*H*)-pyridinyl]-1-(4-fluorophenyl)-1-butanone (Imp. 6). A literature search has shown several analytical methods for analysis of antipsychotic drugs, including haloperidol, in biological fluids (2, 3), spectrophotometric methods for the determination of haloperidol (4), an adsorptive stripping voltammetry assay for determination of haloperidol in bulk form (5), an HPLC method for determination of haloperidol and its metabolites in plasma (6). Although some RP HPLC methods have been developed for the degradation study of haloperidol (7, 8), there are no references in the literature concerning chemometrics approach to the development and validation of the RR RP-HPLC method for simultaneous determination of haloperidol and related compounds specified as impurities. This paper describes the development and validation of a rapid, simple and robust RR RP-HPLC method for simultaneous determination of haloperidol and the above mentioned related substances using a design of experiments (DoE) approach. Development and validation of the method were performed using the experimental design for method optimization and robustness testing. Applying the chemometrics approach enables a relatively limited number of experiments to define factors which affect the chromatographic behavior of investigated substances and to obtain optimum conditions for their analysis.

## EXPERIMENTAL

### *Chemicals and reagents*

Acetonitrile, ACN (gradient grade, Merck, Germany), water (HPLC grade), potassium dihydrogen phosphate, $KH_2PO_4$, (analytical grade, Merck, Germany) and 10 % orthophosphoric acid were used to prepare the mobile phase. Methanol (analytical grade, Merck, Germany) was used as solvent. Phosphate buffer solution (0.025 mol $L^{-1}$) was prepared by dissolving potassium dihydrogen phosphate in water; pH was adjusted to 6.5 with 10 % orthophosphoric acid. Haloperidol, 4-(4-chlorophenyl)-4-hydroxy piperidine (Imp. 1), 4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-hydroxyphenyl)-1-butanone (Imp. 2), 4-fluorobenzoic acid (Imp. 3), *trans*-4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4--fluorophenyl)-1-butanone, *N*-oxide monohydrate (Imp. 4), *cis*-4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone-*N*-oxide (Imp. 5) and 4-[4-(4-chlorophenyl)-3,6-dihydro-1(2*H*)-pyridinyl]-1-(4-fluorophenyl)-1-butanone (Imp. 6) were kindly supplied by Krka d.d., Slovenia.

### *Preparation of standard and test solutions*

Standard stock solutions were prepared by dissolving the respective working standard substances in methanol to obtain the concentration of 200 μg $mL^{-1}$ for haloperidol and 20 μg $mL^{-1}$ for all related compounds (Imp. 1, Imp. 2, Imp. 3, Imp. 4, Imp. 5 and Imp. 6). The standard stock solution was diluted with methanol to obtain working stan-

dard solutions. The test solution contained a mixture of haloperidol and related compounds in a concentration ratio corresponding to ICH recommendations (9). The test solution contained 30 μg $mL^{-1}$ haloperidol and 0.1 μg $mL^{-1}$. Injection of individual working standard solutions (containing only one compound) was used for peak identification. Only chromatograms acquired with the test solution were used in the calculation of chromatographic responses.

### *Apparatus*

Agilent Rapid Resolution HPLC system, 1200 series (consisting of a binary pump SL, a diode array detector SL and column compartment TCC SL) was used. The samples were introduced through a high performance auto sampler SL (HIP-ALS SL). A single UV absorbance was measured at 230 nm. The peak areas were integrated automatically with the Windows NT based LC ChemStation Software. The MODDE 8.0 Software for the design of experiments and optimization (Umetrics, Umea, Sweden) was used for generation and evaluation of the experimental designs.

### *Chromatographic conditions*

Separations were performed using a column Zorbax Eclipse XDB C18 rapid resolution high-throughput (RR HT) 4.6 mm × 50 mm, 1.8 μm particle size, at 25 °C. Injection volume was 10 μL. UV detection was done at 230 nm. All mobile phases were filtered through a 0.2-μm Millipore filter.

### *Validation procedure*

To study the linearity of the response, a series of working standard solutions of 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μg $mL^{-1}$ for haloperidol and of 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 and 0.50 μg $mL^{-1}$ for each related compound were prepared. The linearity of peak area response *vs*. concentration was studied. The correlation graph was constructed by plotting the peak area obtained under optimized conditions. Precision and $e_r$ were assessed using three different working standard solutions (5, 25 and 50 μg $mL^{-1}$ of haloperidol; 0.05, 0.25 and 0.50 μg $mL^{-1}$ of each related compound). Five determinations were carried out for each solution.

For wavelength selection, standard solutions of 30 μg $mL^{-1}$ for haloperidol and 0.1 μg $mL^{-1}$ of each related compound were prepared.

### *Optimization*

Full factorial $2^3$ design was applied to optimize the separation of a mixture of haloperidol and its impurities. Organic phase variation during gradient elution, flow rate and the gradient rise time were investigated at three different levels each.

### *Robustness*

Robustness testing was performed in accordance with Central Composite Circumscribed (CCC) Design. Three basic gradients were tested where the ACN content in the mobile phase was varied from 20 to 80 %, from 20 to 70 % and from 20 to 60% (*V/V*), respectively. The gradient rise time was varied between 5 and 10 min. Flow rate was varied from 1 to 2 mL $min^{-1}$. For estimation of the system response during optimization procedure and robustness testing, resolution ($R_s$) and a chromatographic response function (*CRF*) were used as response factors.

## RESULTS AND DISCUSSION

### *Optimization of separation of a mixture of haloperidol and its impurities*

Haloperidol, Imp. 1, Imp. 2, Imp. 4, Imp. 5 and Imp. 6 have basic properties. Imp. 3 is monoprotic acid. Imp. 2, Imp. 4, Imp. 5 and Imp. 6 are process related impurities and possible degradation products. Imp. 1 and Imp. 3 are hydrolytic products of haloperidol (Fig. 1).

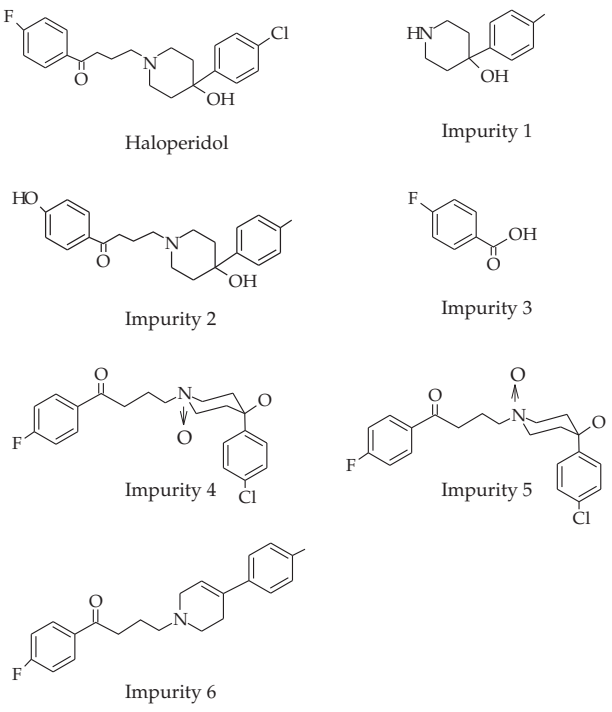



Fig. 1. Chemical structure of haloperidol and its degradation products.

Because of their similar structure, separation of the related substances and haloperidol is very difficult. Similar affinity to the stationary phase, low symmetry of peaks and long retention time characterize the RR RP-HPLC analysis of these substances. The reported RP HPLC methods for simultaneous determination of haloperidol and related compounds describes RP HPLC procedures employing various C18 columns, mobile phases with low pH and addition of triethylamine to the mobile phase to improve the sharpness of the haloperidol peak (7, 8). As the objective of the investigation was to resolve haloperidol and related impurities in a short analysis time with no compromise in resolution, sensitivity and robustness, the Eclipse XDB C18 rapid resolution high-throughput, 4.6 mm × 50 mm, 1.8 μm particle size column was used. The Eclipse XDB column is extra densely bonded and double end-capped and it can be used over a wide pH range. Columns with short (50 mm) length and 1.8 μm particle size provide very high resolution in a short analysis time (10, 11). During preliminary investigations, chromatographic behavior of haloperidol and its related substances was examined using the mobile phase of different polarities. Several mobile phases containing 0.025 mol $L^{-1}$ phosphate buffer systems with different pH values were tested. It was observed that pH variations between 2.5 and 6.5 led to an increase of the resolution between all investigated compounds which behave as bases, while the resolution between peaks of Imp. 2 and Imp. 3 decreased with increasing pH, as expected (8). Considering the p$K_a$ value of haloperidol (p$K_a$ = 8.3) and structure similarity of related compounds, pH value of 6.5 was chosen. Several mobile phases containing acetonitrile and buffer at pH 6.5 were examined where the composition of the organic phase was varied from 20:80 to 80:20 (organic phase/buffer, *V/V*). The best result was obtained using the mobile phase containing 60:40 (organic phase/buffer, *V/V*), but the peaks of Imp. 1/Imp. 2 and Imp. 4/Imp. 5, were not very well resolved. Thus, that gradient elution seemed necessary in order to achieve sufficient resolution. We have used a full factorial $2^3$ design for simultaneous three-factor optimization of the separation of a mixture of haloperidol and its impurities. The method is based on modeling the resolution ($R_s$) using a polynomial of three factors according to a rectangular design. The three factors varied were: organic phase variation during gradient elution ($x_1$), flow rate ($x_2$) and the gradient rise time ($x_3$), investigated at three different levels each. The experimental domain was defined and a zero-level (center), in which all variables are fixed at their mean value, was included in order to minimize the risk of missing non-linear relationships (12). A minimum obtained value of individual $R_s$ values of 2.5 was used as a selection criterion. Eleven experiments were carried out and $R_s$ values of all consecutive peak pairs were calculated. The total number of detected peak pairs was six: (1) Imp. 1/Imp. 2; (2) Imp. 2/Imp. 3; (3) Imp. 3/Hal; (4) Hal/Imp. 4; (5) Imp. 4/Imp. 5; (6) Imp. 5/Imp. 6. The elution order did not change with the mobile phases tested. The $R_s$ values of obtained peak pairs in gradient elution using different composition of the mobile phase and different gradient time are presented in Table I.

In the full factorial $2^3$ experimental design, a linear mathematical model of the measured response is often applied for the evaluation of the influence of investigated factors. An often used linear model is:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \quad (1)$$

*Table I. The* $R_s$ *values of eluted peak pairs*

| $x_1$ (%)[b] | $x_2$ (mL min$^{-1}$) | $x_3$ (min) | Eluted peak pairs[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | CRF |
| | | | $R_s$ | | | | | | |
| 20 → 60 | 1 | 5 | 1.73 | 17.38 | 19.45 | 17.55 | 1.15 | 6.94 | 11.2 |
| 20 → 80 | 1 | 5 | 1.91 | 16.02 | 20.14 | 15.99 | 1.85 | 7.32 | 43.78 |
| 20 → 60 | 2 | 5 | 1.78 | 14.76 | 22.56 | 14.24 | 1.49 | 7.66 | 11.50 |
| 20 → 80 | 2 | 5 | 2.05 | 12.37 | 16.17 | 12.12 | 2.23 | 6.69 | 45.68 |
| 20 → 60 | 1 | 10 | 2.39 | 14.61 | 19.13 | 14.48 | 2.61 | 7.15 | 15.69 |
| 20 → 80 | 1 | 10 | 3.15 | 13.95 | 17.53 | 13.82 | 2.75 | 6.90 | 49.80 |
| 20 → 60 | 2 | 10 | 2.47 | 13.64 | 11.67 | 13.79 | 2.38 | 5.90 | 23.32 |
| 20 → 80 | 2 | 10 | 2.93 | 14.14 | 14.53 | 14.05 | 2.59 | 6.15 | 39.78 |
| 20 → 70 | 1.5 | 7.5 | 2.53 | 14.63 | 16.08 | 14.40 | 2.61 | 6.33 | 29.25 |
| 20 → 70 | 1.5 | 7.5 | 2.55 | 14.63 | 16.10 | 14.42 | 2.64 | 6.63 | 29.30 |
| 20 → 70 | 1.5 | 7.5 | 2.54 | 14.61 | 16.09 | 14.41 | 2.63 | 6.65 | 29.27 |

[a] For eluted peak pairs refer to the text. [b] Organic phase variation during gradient elution.

where $y$ represents the estimated response, $b_o$ is the average experimental response, coefficients $b_1$, $b_2$, and $b_3$ are the estimated effects of the factors considered. The extent to which these terms affect the performance of the method is called the main effect. The coefficients $b_{12}$, $b_{13}$, $b_{23}$ and $b_{123}$ are called interaction terms. In this way, the factorial design provides information about the importance of interaction between the factors. The number of coefficients is equal to the number of experiments (in our experiment 8). The zero-level experiment was not included in the calculation of coefficients. Also, $b_o$ is the intercept of the linear model, $b_1$, $b_2$ and $b_3$ are the main effects, $b_{12}$, $b_{13}$ and $b_{23}$ are two-factor interactions and $b_{123}$ are a three-factor interaction (13). The values of the obtained coefficients are listed in Table II.

*Table II. Values of the obtained coefficients according to Eq. (1)*

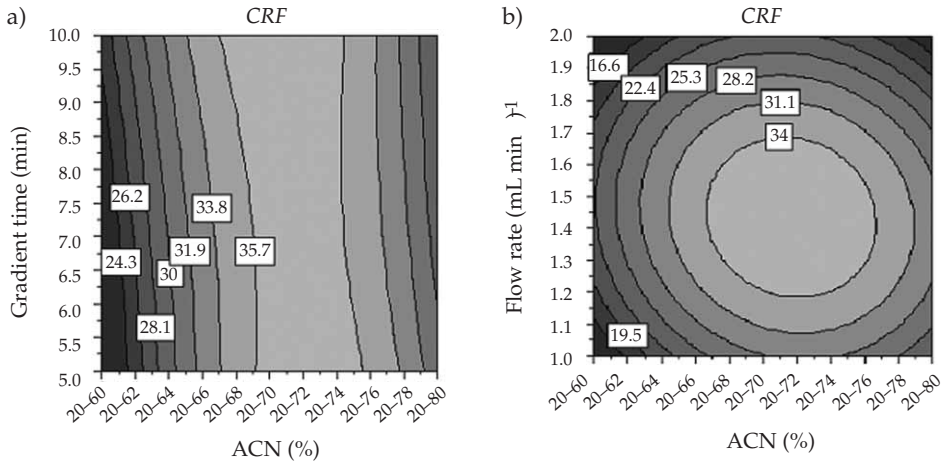
| | $b_o$ | $b_1$ | $b_2$ | $b_3$ | $b_{12}$ | $b_{13}$ | $b_{23}$ | $b_{123}$ |
|---|---|---|---|---|---|---|---|---|
| Imp. 1/Imp. 2 | 2.76 | –1.02 | 0.19 | –0.88 | –0.04 | 0.64 | –0.01 | 0.05 |
| Imp. 2/Imp. 3 | 1.66 | 0.54 | 0.07 | –0.12 | 0.12 | –0.08 | –0.03 | 0.09 |
| Imp. 3/Hal | 2.06 | 0.41 | 0.12 | –0.19 | 0.01 | –0.03 | –0.00 | 0.02 |
| Hal/Imp. 4 | 1.21 | –0.37 | 0.06 | –0.20 | –0.02 | 0.03 | –0.01 | 0.01 |
| Imp. 4/Imp. 5 | 3.22 | –0.91 | 0.13 | –1.08 | –0.05 | 0.45 | –0.08 | 0.07 |
| Imp. 5/Imp. 6 | 1.04 | 0.78 | 0.01 | 0.06 | 0.03 | 0.03 | 0,01 | 0.01 |

Values of coefficients $b_2$ for the first (Imp. 1/Imp. 2) and the fifth (Imp. 4/Imp. 5) peak pairs, and especially the values of coefficients $b_1$ for all peak pairs, demonstrate that separation of the investigated substances as measured by the $R_s$ values is most affected by the organic phase variation during gradient elution ($x_1$) and gradient rise time ($x_3$). Values of the coefficients for the two-factor interaction, $b_{13}$ for the first (Imp. 1/Imp. 2) and the fifth (Imp. 4/Imp. 5) peak pairs, confirmed the main factor effects. Flow rate of the mobile phase had low influence on the investigated system responses. In order to investigate the chromatographic behavior of the investigated substances for the given experimental range and to define the optimum separation conditions, further optimization of the method was performed using response surface methodology (RSM). This is a collection of mathematical and statistical techniques useful for analyzing problems where several independent variables influence a dependent variable or response and the goal is to optimize this response (14). A chromatographic response function (*CRF*) was used for optimizing the separation quality of haloperidol and its impurities in such a way, that maximum resolution with the minimum assay time was obtained. The *CRF* is a coefficient which characterizes the quality of separation in a quantitative manner. Preferably, a flexible function that allows resolution criteria to be specified (15). The corresponding terms in the chromatogram are then compared to these criteria. In this work, a very simple but very useful *CRF* is used:

$$CRF = \sum_{i=1}^{L-1} R_s(i,i+1) \qquad (2)$$

where $R_s(i,i+1)$ is the resolution between peak no. $i$ and peak no. $i+1$.

Minimum value of individual $R_s$ value obtained of 2.5 as a selection criterion was used. The total number of detected peak pairs ($L$) was six. *CRF* values were calculated for all eleven experiments (Table I) and a contour diagram was constructed (Fig. 2). Con-



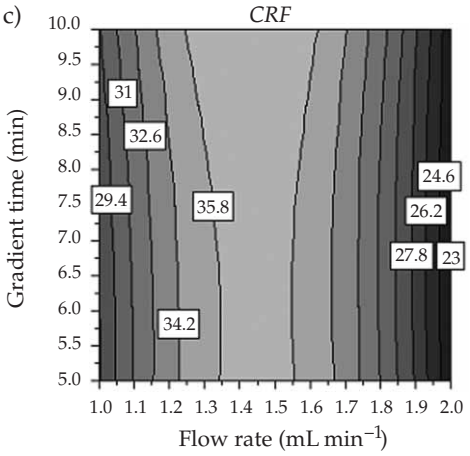



Fig. 2. A contour diagram of the *CRF* as a function of: (a) organic phase variation during gradient elution (ACN, %) and gradient time (min); (b) organic phase variation during gradient elution (ACN, %) and flow rate (mL $min^{-1}$); (c) gradient time (min) and flow rate (mL $min^{-1}$).

tour diagram presents the *CRF* values as a function of two variables while the third is kept constant at zero-level.

The obtained results clearly show that the organic phase variation during gradient elution had the strongest influence on the resolution factor, yielding sufficient resolution: organic phase variation during gradient elution from 20–69 to 20–76 %, *V/V* (see Fig. 2a and 2b), in gradient rise time from 6.5 to 7.5 mL $min^{-1}$ (Figs. 2a and 2c) and in flow rate from 1.3 to 1.6 mL $min^{-1}$ (Fig. 2c). The best result, which corresponds to high *CRF* values was obtained using the mobile phase containing ACN as organic modifier and phosphate buffer pH 6.5, with organic phase variation from 20:80 to 72:28 % *V/V*,

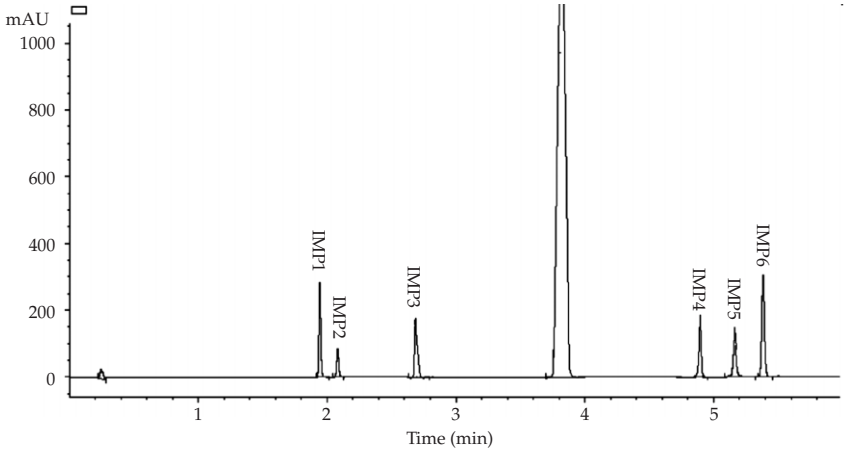



Fig. 3. Representative chromatogram of the test solution [organic phase variation during gradient elution from 20:80 to 72:28% (*V/V*); gradient rise time of 7 min; flow rate of 1.5 mL $min^{-1}$ at 25 °C].

*Table III. Chromatographic parameters for the representative chromatogram*

| | Eluted peaks[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| $t_r$ (min) | 1.93 | 2.07 | 2.68 | 3.77 | 4.88 | 5.16 | 5.38 |
| $k'$ | 1.14 | 1.94 | 1.69 | 2.77 | 3.88 | 4.16 | 4.38 |
| | Eluted peak pairs[b] | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| $R_s$ | 3.19 | 15.0 | 16.67 | 15.09 | 2.76 | 6.48 | |
| α | 1.15 | 1.57 | 1.64 | 1.40 | 1.27 | 1.25 | |

$t_r$ – retention time, $k'$ – capacity factor, α – selectivity, $R_s$ – resolution.
[a] Eluted peaks: (1) Imp. 1; (2) Imp. 2; (3) Imp. 3; (4) haloperidol; (5) Imp. 4; (6) Imp. 5; (7) Imp. 6.
[b] For eluted peak pairs refer to the text.

gradient rise time of 7 min and flow rate of 1.5 mL min$^{-1}$ at 25 °C. The representative chromatogram of the test solution obtained under optimized conditions is represented in Fig. 3.

Resolution between peaks of Imp. 1/Imp. 2 and Imp. 4/Imp. 5 were 3.19, and 2.76, respectively. All respective compounds were clearly separated and their corresponding peaks were sharply developed. Separation was obtained in 5.5 min. The chromatographic parameters for the representative chromatogram are given in Table III.

## *Validation*

After establishing the optimal conditions for separation, linearity, precision, modal recovery, limit of detection and limit of quantitation were determined for all investiga-

*Table IV. Calibration and limiting value parameters*

| | Eluted peaks[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| Parameter | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| a | 0.48 | –0.61 | 0.51 | –5.62 | 0.47 | 0.71 | 4.27 |
| b | 144.8 | 132.5 | 330.3 | 17.56 | 54.03 | 159.7 | 309.9 |
| $R^2$ | 0.9997 | 0.9998 | 0.9998 | 0.9999 | 0.9997 | 0.9998 | 0.9998 |
| *DL* (μg mL$^{-1}$) | 0.027 | 0.027 | 0.031 | 1.16 | 0.035 | 0.027 | 0.020 |
| *QL* (μg mL$^{-1}$) | 0.089 | 0.082 | 0.099 | 3.86 | 0.12 | 0.089 | 0.061 |

[a] Eluted peaks: (1) Imp. 1; (2) Imp. 2; (3) Imp. 3; (4) haloperidol; (5) Imp. 4; (6) Imp. 5; (7) Imp. 6.
a – intercept, b – slope, $R^2$ – coefficient of determination, *DL* – limit of detection, *QL* – limit of quantitation.

ted substances. Limit of detection values were determined at a signal-to-noise ratio of 3 (S/N = 3) and limit of quantitation values at S/N = 10. Important calibration curve parameters: slope (a), intercept (b), coefficient of determination ($R^2$), as well as limit of detection (*DL*) and limit of quantitation (*QL*) are given in Table IV.

Values obtained from precision and $e_r$ determinations indicate that the assay was precise *RSD* range from 1.2 to 1.9 % for haloperidol and from 1.2 to 2.7 % for its related compounds; recovery values from 99.2 to 102.0 % for haloperidol and from 98.9 to 102.4 % for the related compounds were estimated for favourable model. The results are given in Table V.

*Table V. Precision and accuracy of the proposed RR RP-HPLC method*

| | Injected (μg mL$^{-1}$) | Determined (μg mL$^{-1}$) | RSD (%) | Modal recovery (%) |
|---|---|---|---|---|
| Haloperidol | 5 | 5.1 ± 0.1[a] | 1.9 | 102.0 |
| | 25 | 24.8 ± 0.3 | 1.2 | 99.2 |
| | 50 | 50.6 ± 0.7 | 1.4 | 101.2 |
| | Injected (ng mL$^{-1}$) | Determined (ng mL$^{-1}$) | RSD (%) | Modal recovery (%) |
| Imp. 1 | 50 | 49.8 ± 0.6 | 1.2 | 99.6 |
| | 250 | 249.7 ± 3.7 | 1.5 | 99.9 |
| | 500 | 502.5 ± 8.5 | 1.7 | 100.5 |
| Imp. 2 | 50 | 49.4 ± 1.3 | 2.6 | 98.9 |
| | 250 | 249.2 ± 3.6 | 1.4 | 99.7 |
| | 500 | 499.0 ± 7.9 | 1.6 | 99.8 |
| Imp. 3 | 50 | 49.9 ± 0.9 | 1.8 | 99.8 |
| | 250 | 251.5 ± 5.2 | 2.0 | 100.6 |
| | 500 | 495.0 ± 7.8 | 1.6 | 99.0 |
| Imp. 4 | 50 | 51.2 ± 1.2 | 2.3 | 102.4 |
| | 250 | 254.2 ± 3.5 | 1.4 | 101.7 |
| | 500 | 498.2 ± 9.5 | 1.9 | 99.6 |
| Imp. 5 | 50 | 50.8 ± 1.4 | 2.7 | 101.6 |
| | 250 | 249.5 ± 3.9 | 1.6 | 99.8 |
| | 500 | 503.5 ± 10.5 | 2.1 | 100.7 |
| Imp. 6 | 50 | 50.6 ± 1.1 | 2.1 | 101.2 |
| | 250 | 252.2 ± 5.5 | 2.2 | 100.9 |
| | 500 | 498.0 ± 9.9 | 2.0 | 99.6 |

[a] Mean ± SD ($n$ = 5).

*Table VI. Experimental plan for robustness testing*

| | Experiment No. | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Factor | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
| $x_1$ | – | + | – | + | – | + | – | + | 0 | 0 | 0 | – | + | 0 | 0 | 0 | 0 |
| $x_2$ | – | – | + | + | – | – | + | + | 0 | 0 | 0 | 0 | 0 | – | + | 0 | 0 |
| $x_3$ | – | – | – | – | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | – | + |

## *Robustness*

As defined by the ICH (27), robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method parameters. In order to study simultaneous variations of the factors on the considered responses, a multivariate approach using a design of experiments is recommended in robustness testing (17, 18). Central Composite Circumfacited (CCC) design required $2^k + 2k + n = 17$ runs, where $k$ is the number of parameters studied ($k = 3$) and $n$ is the number of central points included ($n = 3$). Three repetitions are generally carried out in order to know the variance of the experimental error and to test the predictive validity of the model (12, 18). The experimental plan is reported in Table VI.

*Table VII. Robustness testing*

| | | Factors levels | | |
|---|---|---|---|---|
| | Factor | (–) | (+) | (0) |
| $x_1$ | Organic phase variation during gradient elution (%) | 20 → 60 | 20 → 80 | 20 → 70 |
| $x_2$ | Flow rate (mL min$^{-1}$) | 1 | 2 | 1.5 |
| $x_3$ | Gradient time (min) | 5 | 10 | 7.5 |

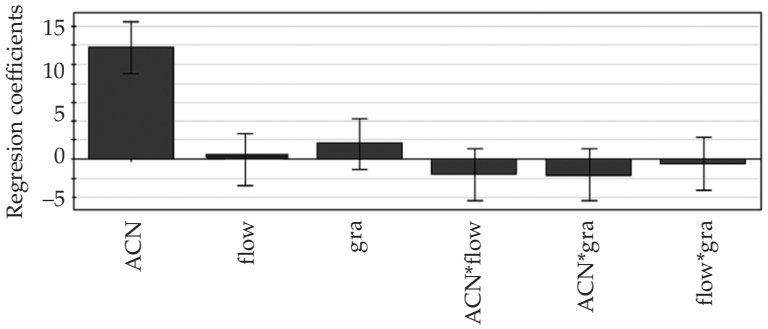



Fig. 4. Regression coefficient plot [ACN: organic phase variation during gradient elution (ACN, %), flow: flow (mL min$^{-1}$), gra: gradient rise time (min)].

The values used at the zero (0), high (+1) and low levels (–1) are shown in Table VII.

The ranges examined were small deviations from the method settings and the considered response was the measured resolution ($R_s$) between each peak pair. *CRF* was calculated for all 17 runs and was used for evaluation of the influence of the factors variation on the separation quality of haloperidol and its impurities. The results are shown in Fig. 4.

The plot consists of bars that correspond to regression coefficients with the magnitude of the effects proportional to regression coefficients. The 95 % confidence limits are expressed using error bars. The results show that the separation under the examined conditions was principally influenced by the organic phase variation during gradient elution and gradient rise time. They both have a positive effect on the *CRF*, which means that an increase of the percentage of acetonitrile in the mobile phase or an increase of the gradient rise time increase the resolution between all peak pairs. The influence of the flow rate of the mobile phase was not significant. No major interactions were found. Statistical analysis of the model gave a $R^2$ value (the fraction of variation of the response that can be explained by the model) of 0.99 and a $Q^2$ value (the fraction of variation of the response that can be predicted by the model) of 0.95. In conclusion, of the analysis confirms that the method is robust for all factors investigated. The statistical analysis showed that the variation of *CRF* was correctly related to the variation of factors, showing a good agreement between experimental and predicted values.

## CONCLUSIONS

The methodology proposed represents an efficient and easily accomplishable approach to resolving the problem of searching for optimum RR RP-HPLC conditions. The linear model obtained demonstrates a strong influence of the organic phase variations during gradient elution and smaller, but significant, influence of the gradient rise time on the resolution between investigated substances. Valuable information about the robustness of the method was obtained by CCC design, using resolution as an important component of *CRF*. The proposed RR RP-HPLC method permits simultaneous determination of haloperidol and its related compounds, specified as impurities, due to good separation and resolution of the chromatographic peaks and robustness towards reasonable changes in chromatographic parameters. The method is a simple, rapid and robust assay for impurity determination and can provide adequate linearity, precision and relative error. The developed method allows determination of haloperidol, its purity and level of impurities in drug substances. Thus, purity of the active substance, level of impurities, and a total chromatographic purity can be determined in a single analysis.

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*S A Ž E T A K*

## Uporaba kemometrije za razvoj i validaciju RP-HPLC metode za simultano određivanje haloperidola i srodnih spojeva

RUMENKA PETKOVSKA i ANETA DIMITROVSKA

Razvijena je i validirana metoda reverzno-fazne tekućinske kromatografije visoke učinkovitosti i brze rezolucije (RR RP-HPLC) za simultano određivanje haloperidola i srodnih spojeva. U tu svrhu ispitivana je smjesa ljekovite tvari haloperidola i šest srodnih spojeva u omjeru 300:1. Za optimiranje metode korišten je eksperimentalni dizajn ($2^3$ faktorijalni dizajn) i testiranje robustnosti (Central *Composite Circumscribed design*). Tri faktora: variranje organske faze za eluaciju, brzina protoka i vrijeme uspostave gradijenta eluensa bile su nezavisne varijable. Za procjenu odgovora sustava za vrijeme optimizacije i testiranje robustnosti, korištene su razlučivanje ($R_s$) i funkcija kromatografskog odziva (*CRF*). Mobilna faza tijekom kromatografije bila je fosfatni pufer pH 6,5 i acetonitril kao organska faza. Razdvajanje je postignuto pomoću gradijenta eluacije (udio organske faze linearno se mijenjao od 20 do 72%) tijekom 7 min. Za rad je upotrebljena kolona Zorbax Eclipse XDB C18 Rapid Resolution HT kolona, dimenzije 4,6 mm × 50 mm, veličine čestica 1,8 μm. Kromatografija je provedena pri 25 °C, uz protok eluensa 1,5 mL $min^{-1}$ i UV detekciju na 230 nm. Vrijeme kromatografskog razdvajanja bilo je 5,5 min, a ukupno vrijeme potrebno za kromatografiju 7,0 min. Metoda je u potpunosti validirana.

*Ključne riječi*: haloperidol, onečišćenja, RP-HPLC, validacija, eksperimentalni dizajn

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