Development and validation of the HPLC/UV-procedure of secnidazole determination

Secnidazole is one of antiprotozoal medicines from the group of 5-nitroimidazoles, the method of HPLC with different types of detection is widely used for secnidazole determination.

**Aim.** To develop the HPLC/UV-procedure of secnidazole quantification with application of the system of a "MiLiChrome® A-02" HPLC-analyzer and carry out the step-by-step validation of the procedure developed.

**Results and discussion.** The specificity of the chromatographic conditions proposed was confirmed in relation to other medicines of the group of 5-nitroimidazoles (metronidazole, tinidazole, ornidazole and nimorazole). The retention time for secnidazole was 8.16 min. 0.01 M solution of hydrochloric acid was proposed for preparation of the reference and mobile solutions, and Eluent B was used as the mobile phase components. The HPLC microcolumn with the size of Ø2 × 75 mm and the ProntoSIL 120-5-C18 AQ reversed phase, 5 μm (BISCHOFF Analysentechnik und -geräte GmbH, Germany) was used as an analytical column. The analysis was performed at 40 °C and the flow rate of 100 μl/min. The mobile phase was run in the gradient elution mode, namely from 5 % to 100 % of Eluent B for 40 min, then 100 % of Eluent B for 3 min. Detection was performed at 277 nm.

**Conclusions.** A new procedure of the secnidazole quantitative determination by the method of HPLC/UV has been developed. Its validation has been carried out, and acceptability for its application has been shown.

**Key words:** secnidazole; high-performance liquid chromatography; validation

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Secnidazole is one of antiprotozoal medicines from the group of 5-nitroimidazoles, for determination of which high-performance liquid chromatography (HPLC) with different types of detection is widely used.

**Aim.** To develop the HPLC/UV-procedure of secnidazole quantification with application of the system of a "MiLiChrome® A-02" HPLC-analyzer and carry out the step-by-step validation of the procedure developed.

**Results and discussion.** The specificity of the chromatographic conditions proposed was confirmed in relation to other medicines of the group of 5-nitroimidazoles (metronidazole, tinidazole, ornidazole and nimorazole). The retention time for secnidazole was 8.16 min. 0.01 M solution of hydrochloric acid was proposed for preparation of the reference and mobile solutions, and Eluent B was used as the mobile phase components. The HPLC microcolumn with the size of Ø2 × 75 mm and the ProntoSIL 120-5-C18 AQ reversed phase, 5 μm (BISCHOFF Analysentechnik und -geräte GmbH, Germany) was used as an analytical column. The analysis was performed at 40 °C and the flow rate of 100 μl/min. The mobile phase was run in the gradient elution mode, namely from 5 % to 100 % of Eluent B for 40 min, then 100 % of Eluent B for 3 min. Detection was performed at 277 nm.

**Conclusions.** A new procedure of the secnidazole quantitative determination by the method of HPLC/UV has been developed. Its validation has been carried out, and acceptability for its application has been shown.

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Результаты и их обсуждение. Специфичность предлагаемых хроматографических условий подтверждена в отношении других препаратов из группы 5-нитроimidазолов (метронидазола, тинидазола, орнидазола и ниморазола). Время удерживания для секнидазола составляет 8,16 мин; 0,01 М раствор хлористоводородной кислоты был предложен для приготовления раствора сравнения и модельных растворов при разработке ВЭЖХ/УФ-методики количественного определения секнидазола. Для доказательства возможности применения предлагаемой методики в дальнейшем анализе валидация была проведена в вариантах метода калибровочного графика и метода стандарта. Такие валидационные параметры, как стабильность, линейность, калибровочная модель, правильность и прецизионность были оценены с помощью модельных растворов.

Экспериментальная часть. ВЭЖХ/УФ-анализ проводили с использованием жидкостного хроматографа высокоового давления MilLiChrome® A-02 (EcoNova, Россия). В качестве компонентов подвижной фазы использовали элюент A (0,2 М LiClO₄, 0,005 М HClO₄) и элюент B (вода, 75 мм). В качестве аналитической колонки использовали ВЭЖХ-микроколонку размером Ø2 × 75 мм с обращенной фазой ProntoSIL 120-5 C18 AQ, 5 мкм (BISCHOFF Analysentechnik und -geräte GmbH, Германия). Анализ проводили при 40 °C и скорости потока 100 мкл/мин. Мобильная фаза подавалась в режиме градиентного элюирования – от 5% до 100% элюента В в течение 40 мин, затем 100 % элюента В в течение 3 мин. Детектирование проводили при 277 нм.

Выводы. Разработана новая методика количественного определения секнидазола методом ВЭЖХ/УФ. Проведена ее валидация и показана приемлемость для применения.

Ключевые слова: секнидазол; высокоэффективная жидкостная хроматография; валидация

Secnidazole is one of the antiprotazoal medicines from the group of 5-nitroimidazoles, it is characterized by a prolonged serum half-life [1, 2] and widely used for the treatment of infectious diseases caused by Trichomonas, Lamblia, Leishmania, etc. [3-10]. Chemically, secnidazole is 1-(2-methyl-5-nitroimidazol-1-yl)propan-2-ol and has the structural formula as shown in Figure.

For secnidazole determination the method of HPLC with different types of detection is widely used, it provides high selectivity and sensitivity of analysis [11-20]. The chemical structure of secnidazole allows using direct UV-spectrophotometry for its quantification, it was previously confirmed by us [21].

The aim of our paper is to develop the HPLC/UV-procedure of secnidazole quantification with application of the system of "MiLiChrome® A-02" HPLC-analyzer [22] and carry out the step-by-step validation of the procedures developed in the variants of the method of the calibration curve (MCC) and the method of standard (MS) in order to choose the optimal variant for further application in analytical toxicology.

Results and discussion

Since secnidazole is readily soluble and rather stable [21] in solutions of diluted mineral acids 0.01 M solution of hydrochloric acid was proposed for preparation of the reference and model solutions in developing the HPLC/UV-procedure of secnidazole quantification. Under these conditions The pH of the solutions was met the requirements to the samples injected to the "MiLiChrome® A-02" HPLC-analyzer [22].

Previously the specificity of the chromatographic conditions proposed was confirmed in relation to other medicines of the group of 5-nitroimidazoles (metronidazole, tinidazole, ornidazole and nimoazole) [23].

The retention time for secnidazole was 8.16 min, in contrast to metronidazole (5.95 min), tinidazole (9.13 min), ornidazole (10.18 min) and nimoazole (14.12 min).

To prove the possibility of application of the procedure proposed in further analysis its validation was carried out in the variants of the method of the calibration curve and the method of standard [28, 29].

Such validation parameters as in-process stability, linearity/calibration model, accuracy and precision (repeatability) were estimated using model solutions.

The validation method by model solutions according to Scheme 1 suggested by us [28] allows assessing the suitability of the actual analytical procedure for further work.

The validation provides application of the normalized coordinates, i.e. transition from the equation $A_i = b_i \cdot C_i + a_i$ to the equation $Y_i = b_i \cdot X_i + a_i$, it allows to calculate the validation characteristics, which do not depend on the analyte, and peculiarities of the method of analysis [30].

The secnidazole concentration in the model solution for the point of 100 % in the normalized coordinates $C_{100\%}^{\text{model}}$ was chosen as the concentration provided the "signal/noise" ratio at the level of 40 [28].

For normalization of the experimental data obtained the reference solution with the analyte concentration of $C_{\text{refm}}^{\text{model}} = C_{100\%}^{\text{model}}$ was used.

The analytical ranges $D$ of the methods application were 25-125 %, 25-150 % and 25-175 %; the number of concentration levels $g$ equaled 5, 6 or 7, respectively, in constant increments of 25 %.

Acceptability criteria for validation parameters were formed on the basis of systematic application of the "insignificance concept" [30, 31] and proceeding from...
Scheme 1. The validation stages of the HPLC/UV-procedures for secnidazole determination
the value of extreme uncertainty $\Delta_{\text{As}}$, which equaled 20% for the method in analytical toxicology [32, 33].

In the MCC acceptability criteria for linear dependence and precision were found proceeding from the equality of uncertainty of plotting the calibration curve $\Delta_{\text{cal}}$ and uncertainty of analysis of the sample to be analysed $\Delta_{\text{sample}}$.

Acceptability criteria for validation parameters were calculated according to two approaches.

**Approach 1:** uncertainty of the analyte quantification in model solutions $\Delta_{\text{model}}$ is equal to uncertainty of the sample preparation procedure:

\[
\max \Delta_{\text{model}} = \frac{\max \Delta_{\text{As}}}{\sqrt{2}} = 0.707 \cdot \max \Delta_{\text{As}} = 0.707 \cdot 20.00 \% = 14.14 \%;
\]

\[
\max \Delta_{\text{cal}} = \max \Delta_{\text{model}} = \frac{\max \Delta_{\text{model}}}{\sqrt{2}} = (1)
\]

\[
= 0.707 \cdot \max \Delta_{\text{As}} = 0.707 \cdot 14.14 \% = 10.00 \%;
\]

\[
\max \delta_{\text{model}} = 0.32 \cdot \max \Delta_{\text{As}} = 4.52 \%;
\]

**Approach 2:** uncertainty of the analyte quantification in model solutions $\Delta_{\text{model}}$ is insignificant compared to the total uncertainty $\Delta_{\text{As}}$:

\[
\max \Delta_{\text{model}} = 0.32 \cdot \max \Delta_{\text{As}} = 6.40 \%;
\]

\[
\max \Delta_{\text{cal}} = \max \Delta_{\text{sample}} = \frac{\max \Delta_{\text{As}}}{\sqrt{2}} = (2)
\]

\[
= 0.707 \cdot \max \Delta_{\text{As}} = 0.707 \cdot 6.40 \% = 4.52 \%;
\]

\[
\max \delta_{\text{model}} = 0.32 \cdot \max \Delta_{\text{As}} = 2.05 \%.
\]

**Validation results.** In-process stability of secnidazole in the model solution was verified by chromatographing the reference solution immediately and in 1, 12, 24 and 48 hours after its preparation, and the systematic error $\sigma_{\text{model stability}}$ was calculated and assessed (Tab. 1). In-process stability of secnidazole in model solutions was satisfied the acceptability criteria for all periods of time only for both Approach 1 and Approach 2.

To determine linearity/calibration model the model solutions 1-7 were analyzed within 1 run, the correlation coefficient $R_{\text{model}}$, rest standard deviation $RSD_{\text{model}}$, as well as the absolute term $\sigma_{\text{model}}$ (if necessary) were calculated and assessed (Tab. 2).

To estimate precision (repeatability) and accuracy:

**MCC:** the concentrations of model solutions 1-7 were calculated using the linear dependence obtained, and the values “found/given” $RR_{\text{model}}$ were used to determine the confidence interval $\Delta_{\text{bg}}$ and the systematic error $\sigma_{\text{model}}$, respectively (Tab. 3);

**MS:** the ratios $Z_{\text{model}}$ for the model solutions 1-7 were calculated and used to determine the confidence interval $\Delta_{\text{bg}}$ and the systematic error $\sigma_{\text{model}}$, respectively (Tab. 4).

The values of the confidence interval and the systematic error were compared with the corresponding acceptability criteria.

The total results of validation allow making the conclusion about acceptable linearity, accuracy and precision of the HPLC/UV-procedure of the secnidazole quantitative determination in the variant of MCC and MS for all ranges of the method application and for both approaches to acceptability estimation. It gives us the possibility to recommend this procedure for further application in analytical toxicology with the purpose of development of the methods for analysis of biological liquids for secnidazole quantification.

For the most cases the procedures in the variant of MCC are characterized by the better values of precision and accuracy than for the variant of MS. That makes the variant of MCC to be optimal for analysis.

**Experimental part**

**Reagents and chemicals.** Secnidazole was of pharmacopeial purity. Acetonitrile CHROMASOLV®Plus for HPLC and perchloric acid (70%, puriss. p.a., ACS reagent) were purchased from Sigma-Aldrich Co. LLC (USA), lithium perchlorate trihydrate was purchased from Panreac Química S.L.U. (Spain).

**The mobile phase preparation.** *Eluent A* (0.2 M LiClO$_4$ – 0.005 M HClO$_4$) and *Eluent B* (acetonitrile) were used as the components of the mobile phase. *Solution 1* and *Solution 2* were obtained for *Eluent A* preparation.

*Solution 1* (4.1 M LiClO$_4$ aqueous solution): 330.00 g of LiClO$_4$ · 3H$_2$O were dissolved in 450 ml of bidistilled water while stirring and heating to 50 °C, the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
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<tbody>
<tr>
<td>$S_{\text{model}}$ stability</td>
<td>0.013052</td>
</tr>
<tr>
<td>$\sigma_{\text{model stability}}$ stability</td>
<td>0.000041</td>
</tr>
<tr>
<td>$\sigma_{\text{model stability}}$, % $\leq \max \sigma_{\text{model}}$</td>
<td>0.31</td>
</tr>
<tr>
<td>Approach 1</td>
<td>≤ 4.52 %</td>
</tr>
<tr>
<td>Approach 2</td>
<td>≤ 2.05 %</td>
</tr>
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</table>
solution obtained was cooled to ambient temperature and transferred to a 500.0 ml volumetric flask, the solution was diluted to the volume with the same solvent and then filtered through the Millex® HA Filter membrane filter (the particle size of 0.45 µm, mixed cellulose esters, PVC housing) purchased from Merck Millipore Corporation (USA).

Solution 2 (4 M LiClO$_4$ solution in 0.1 M HClO$_4$ solution): 2.2 ml of HClO$_4$ was measured by a 5.0 ml pipette into a 250.0 ml volumetric flask; the solution was diluted to the volume with bidistilled water.

Instrumentation and chromatographic conditions. The HPLC/UV analyses were performed using a MilLiChrome® A-02 high pressure liquid chromatograph (EcoNova, Russia) equipped with a double syringe gradient pump, an autosampler (with the sample volume of 0-99 μl), a column oven (35-90 °C) and a double-beam multiwave UV-spectrophotometer as a detector. The Analitika-Chrom® software (Analitika SPF, Ukraine) was used for integration and processing of chromatograms. The HPLC microcolumn with the size of Ø2 ´ 75 mm and the ProntoSIL 120-5-C18 AQ reversed phase, 5 μm (BISCHOFF Analysentechnik und -geräte GmbH, Germany) was used as an analytical column. The analysis was performed at 40 °С and the flow rate of 100 μl/min. The mobile phase was run in the gradient elution mode, namely from 5 % to 100 % of Eluent B for 40 min, then 100 % of Eluent B for 3 min. Detection was performed at 277 nm. The volume of injection was 2 μL.

<table>
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<tr>
<th>Parameter</th>
<th>Values</th>
<th>Acceptability criterion</th>
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<tr>
<td>$b^\text{model}$</td>
<td>0.981</td>
<td>–</td>
</tr>
<tr>
<td>$s_0^\text{model}$</td>
<td>0.017</td>
<td>–</td>
</tr>
<tr>
<td>$\sigma^\text{model}$</td>
<td>–1.115</td>
<td>–</td>
</tr>
<tr>
<td>$s_\sigma^\text{model}$</td>
<td>1.947</td>
<td>–</td>
</tr>
<tr>
<td>$RSD_0^\text{model}$</td>
<td>2.304</td>
<td>≤ 4.96 %</td>
</tr>
<tr>
<td>$R_s^\text{model}$</td>
<td>0.9992</td>
<td>≥ 0.9958</td>
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<tr>
<td>$b^\text{model}$</td>
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<td>–</td>
</tr>
<tr>
<td>$s_0^\text{model}$</td>
<td>0.020</td>
<td>–</td>
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<tr>
<td>$\sigma^\text{model}$</td>
<td>0.006</td>
<td>–</td>
</tr>
<tr>
<td>$s_\sigma^\text{model}$</td>
<td>1.990</td>
<td>–</td>
</tr>
<tr>
<td>$RSD_0^\text{model}$</td>
<td>2.138</td>
<td>≤ 4.69 %</td>
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<tr>
<td>$R_s^\text{model}$</td>
<td>0.9991</td>
<td>≥ 0.9950</td>
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<td>$b^\text{model}$</td>
<td>0.956</td>
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<td>0.030</td>
<td>–</td>
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<tr>
<td>$\sigma^\text{model}$</td>
<td>0.483</td>
<td>–</td>
</tr>
<tr>
<td>$s_\sigma^\text{model}$</td>
<td>2.519</td>
<td>–</td>
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<tr>
<td>$RSD_0^\text{model}$</td>
<td>2.402</td>
<td>≤ 4.25 %</td>
</tr>
<tr>
<td>$R_s^\text{model}$</td>
<td>0.9985</td>
<td>≥ 0.9942</td>
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Table 3

<table>
<thead>
<tr>
<th>Factual concentration of secnidazole in model solution ( (C_{\text{reference}} = 8 , \mu g/mL) )</th>
<th>Peak area ( \chi_{\text{model},i} ), %</th>
<th>Found in % to standard peak area ( \chi_{\text{ref},i} ), %</th>
<th>Calculated concentration of secnidazole in model solution ( \chi_{\text{f,calc},i}, % )</th>
<th>( RR_{\text{model},i}, % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>25</td>
<td>0.003137</td>
<td>24.02</td>
<td>25.62</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>0.006261</td>
<td>47.94</td>
<td>50.00</td>
</tr>
<tr>
<td>6</td>
<td>75</td>
<td>0.009367</td>
<td>71.73</td>
<td>74.25</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>0.012997</td>
<td>99.52</td>
<td>102.58</td>
</tr>
<tr>
<td>10</td>
<td>125</td>
<td>0.015377</td>
<td>117.74</td>
<td>121.15</td>
</tr>
<tr>
<td>12</td>
<td>150</td>
<td>0.018979</td>
<td>145.32</td>
<td>149.26</td>
</tr>
<tr>
<td>14</td>
<td>175</td>
<td>0.022551</td>
<td>172.67</td>
<td>177.14</td>
</tr>
</tbody>
</table>

\( \sigma_{\text{model,i}}, \% = |100 - RR_{\text{model,i}}| \leq \max \sigma_{\text{model}} \)

Approach 1

\( \leq 4.52 \% \) satisfied satisfied satisfied

Approach 2

\( \leq 2.05 \% \) satisfied satisfied satisfied

\( \Delta_{\text{RR},i}, \% = RSD_{\text{RR},i} \cdot t(95 \%; g - 1) \leq \max \Delta_{\text{RR}} \)

Approach 1

\( \leq 10.00 \% \) satisfied satisfied satisfied

Approach 2

\( \leq 4.52 \% \) satisfied satisfied unsatisfied

Table 4

<table>
<thead>
<tr>
<th>Actual concentration of secnidazole in model solution ( (C_{\text{model,reference}} = 8 , \mu g/mL) )</th>
<th>Peak area ( \chi_{\text{model},i} ), %</th>
<th>Found in % to standard peak area ( \chi_{\text{ref},i} ), %</th>
<th>( Z_{\text{model},i}, % )</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>25-175</td>
<td>25-150</td>
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</table>

\( \sigma_{\text{model,i}}, \% = |100 - Z_{\text{model,i}}| \leq \max \sigma_{\text{model}} \)

Approach 1

\( \leq 4.52 \% \) satisfied satisfied satisfied

Approach 2

\( \leq 2.05 \% \) unsatisfied unsatisfied unsatisfied

\( \Delta_{Z_{i},i}, \% = RSD_{Z_{i}} \cdot t(95 \%; g - 1) \leq \max \Delta_{Z_{i}} \)

Approach 1

\( \leq 14.14 \% \) satisfied satisfied satisfied

Approach 2

\( \leq 6.40 \% \) satisfied satisfied satisfied
Weighing was carried out using an AN100 digital analytical balance (AXIS, Ukraine) with \( \delta = 0.0001 \) g.


**Reference and model solutions** (Scheme 2).
The stock solutions 1 and 2 (100 µg/mL) were prepared by dissolving 50.0 mg of secnidazole in distilled water; the solutions were diluted to 500.0 mL with the same solvent. The reference solution (8 µg/mL) was prepared by diluting 4.00 mL of the stock solution 1 to 500.0 mL with distilled water. The stock solution 2 was diluted with distilled water to prepare the model solutions 1-7 having concentrations of 2; 4; 6; 8; 10; 12 and 14 µg/mL, respectively.

When carrying out experiments each solution (except the in-process stability study) was chromatographed 3 times or, as required, following the requirements to repeatability of peak areas for replicate injections offered by us [28] – the relative standard deviation of the mean \( RSD_{\text{nom}} \) calculated towards the nominal value of peak area \( S_{\text{nom}} \) should not exceed:

\[
RSD_{\text{nom}} = \frac{S}{S_{\text{nom}}} \cdot 100 \% \leq \max RSD_{\text{nom}} = \frac{0.1 \cdot \max \Delta_{\Delta S}}{t(95\% ; n - 1)}
\]

where: \( S_{\text{nom}} \) – is the mean peak area obtained when analyzing model solution 1. The mean values were used in further calculations.

**Conclusions**

A new procedure of the secnidazole quantitative determination by the method of HPLC/UV has been developed. Its validation by such parameters as stability, linearity, accuracy and precision in the variants of the method of the calibration curve and the method of standard has been carried out and acceptability for its application has been shown.

**Conflict of Interests:** authors have no conflict of interests to declare.

**References**

References


