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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 model solutions in developing the HPLC/UV-procedure of secnidazole quantification. 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. Such validation parameters as in-process stability, linearity/calibration model, accuracy and precision (repeatability) were estimated using model solutions.

Experimental part. The HPLC/UV analyses were performed using a MiLiChrome® A-02 high pressure liquid chromatograph (EcoNova, Russia). *Eluent A* (0.2 M LiClO₄ – 0.005 M HClO₄) and *Eluent B* (acetonitrile) were 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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Розробка та валідація ВЕРХ/УФ-методики визначення секнідазолу

Секнідазол є одним з антипротозойних препаратів з групи 5-нітроїмідазолів, для визначення якого широко використовується метод ВЕРХ з різними типами детекції.

Мета. Розробити ВЕРХ/УФ-методику кількісного визначення секнідазолу з використанням системи ВЕРХ-аналізатора «MiLiChrome® A-02» і провести поетапну валідацію розробленої методики.

Результати та їх обговорення. Специфічність запропонованих хроматографічних умов підтверджено відносно інших препаратів з групи 5-нітроїмідазолів (метронідазолу, тінідазолу, орнідазолу і німоразолу). Час утримування для секнідазолу становить 8,16 хв; 0,01 М розчин хлористоводневої кислоти було запропоновано для приготування розчину порівняння і модельних розчинів при розробці ВЕРХ/УФ-методики кількісного визначення секнідазолу. Для доказу можливості застосування запропонованої методики в подальшому аналізі була проведена її валідація у варіантах методу калібрувального графіка і методу стандарту. Такі валідаційні параметри, як стабільність, лінійність/калібрувальна модель, правильність і прецизійність були оцінені за допомогою модельних розчинів.

Експериментальна частина. ВЕРХ/УФ-аналіз проводили з використанням рідинного хроматографа високого тиску MiLiChrome® A-02 (EcoNova, Росія). Як компоненти рухомої фази використовували елюент А (0,2 М LiClO₄ – 0,005 М HClO₄) і елюент В (ацетонітрил). Як аналітичну колонку використовували ВЕРХ-мікроколонку розміром Ø2 × 75 мм з оберненою фазою ProntoSIL 120-5-C18 AQ, 5 мкм (BISCHOFF Analysentechnik und -geräte GmbH, Німеччина). Аналіз проводили при 40 °C і швидкості потоку 100 мкл/хв. Мобільна фаза подавалася в режимі градієнтного елюювання – від 5 % до 100 % елюенту В впродовж 40 хв, потім 100 % елюенту В впродовж 3 хв. Детектування проводили при 277 нм.

Висновки. Розроблено нову методику кількісного визначення секнідазолу методом ВЕРХ/УФ. Проведена її валідація і показана прийнятність для застосування.

Ключові слова: секнідазол; високоефективна рідинна хроматографія; валідація

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Разработка и валидация ВЭЖХ/УФ-методики определения секнидазола

Секнидазол является одним из антипротозойных препаратов из группы 5-нитроимидазолов, для определения которого широко используется метод ВЭЖХ с различными типами детекции.

Цель. Разработать ВЭЖХ/УФ-методику количественного определения секнидазола с использованием системы ВЭЖХ-анализатора «MiLiChrome® A-02» и провести поэтапную валидацию разработанной методики.

Результаты и их обсуждение. Специфичность предлагаемых хроматографических условий подтверждена в отношении других препаратов из группы 5-нитроимидазолов (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 высокого давления MiLiChrome® A-02 (EcoNova, Россия). В качестве компонентов подвижной фазы использовали элюент А (0,2 М LiClO₄ – 0,005 М HClO₄) и элюент В (ацетонитрил). 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 °С 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 antiprotozoal 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*, *Lambliа*, *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 a “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 solu-

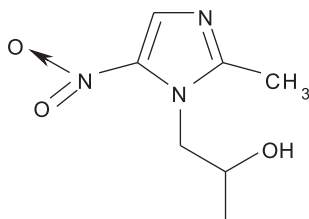


Fig. The chemical structure of secnidazole

tions 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 nimorazole) [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 nimorazole (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.

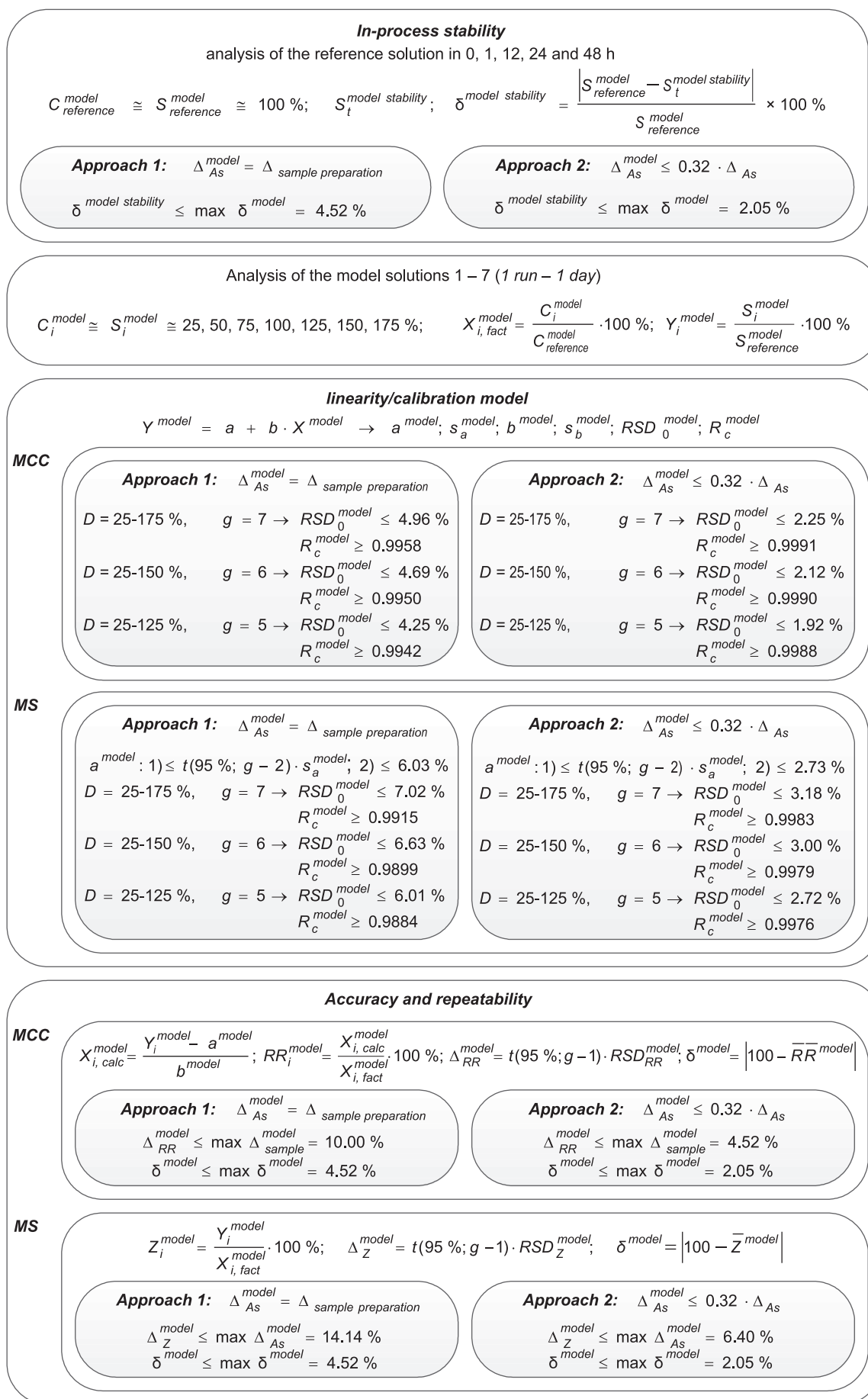
The validation provides application of the normalized coordinates, i. e. transition from the equation $A_i = b_1 \cdot C_i + a_1$ to the equation $Y_i = b_2 \cdot X_i + a_2$, it allows to calculate the validation characteristics, which do not depend on the analyte, and peculiarities of the method of analysis [30].

The secnidazole concentration in the model solution for the point of 100 % in the normalized coordinates $C_{100\%}^{model}$ 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 $C_{reference}^{model} = C_{100\%}^{model}$ 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 Δ_{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 Δ_{cal} and uncertainty of analysis of the sample to be analysed Δ_{sample} .

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

Approach 1: uncertainty of the analyte quantification in model solutions Δ_{As}^{model} is equal to uncertainty of the sample preparation procedure:

$$\begin{aligned} \max \Delta_{As}^{model} &= \frac{\max \Delta_{As}}{\sqrt{2}} = 0.707 \cdot \max \Delta_{As} = \\ &= 0.707 \cdot 20.00 \% = 14.14\%; \end{aligned}$$

$$\begin{aligned} \max \Delta_{cal}^{model} &= \max \Delta_{sample}^{model} = \frac{\max \Delta_{As}^{model}}{\sqrt{2}} = \\ &= 0.707 \cdot \max \Delta_{As}^{model} = 0.707 \cdot 14.14 \% = 10.00\%; \end{aligned} \quad (1)$$

$$\max \delta^{model} = 0.32 \cdot \max \Delta_{As}^{model} = 4.52 \%;$$

Approach 2: uncertainty of the analyte quantification in model solutions Δ_{As}^{model} is insignificant compared to the total uncertainty Δ_{As} :

$$\max \Delta_{As}^{model} = 0.32 \cdot \max \Delta_{As} = 0.32 \cdot 20.00 \% = 6.40 \%;$$

$$\begin{aligned} \max \Delta_{cal}^{model} &= \max \Delta_{sample}^{model} = \frac{\max \Delta_{As}^{model}}{\sqrt{2}} = \\ &= 0.707 \cdot \max \Delta_{As}^{model} = 0.707 \cdot 6.40 \% = 4.52 \%; \end{aligned} \quad (2)$$

$$\max \delta^{model} = 0.32 \cdot \max \Delta_{As}^{model} = 0.32 \cdot 6.40 \% = 2.05 \%.$$

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 $\sigma^{model\ stability}$ 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_c^{model} , rest standard deviation RSD_0^{model} , as well as the absolute term a^{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_i^{model} were used to determine the confidence interval Δ_{RR}^{model} and the systematic error σ^{model} , respectively (Tab. 3);

MS: the ratios Z_i^{model} for the model solutions 1-7 were calculated and used to determine the confidence interval Δ_z^{model} and the systematic error σ^{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 pharmacopoeial 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 $\text{LiClO}_4 - 0.005 \text{ M HClO}_4$) 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 $\text{LiClO}_4 \cdot 3\text{H}_2\text{O}$ were dissolved in 450 ml of bidistilled water while stirring and heating to 50 °C, the

Table 1

The results of in-process stability verification for secnidazole in model solutions

Parameter	Values					
	0 h	1 h	12 h	24 h	36 h	48 h
$S^{model\ stability}$	0.013052	0.013011	0.013089	0.013094	0.012987	0.012963
$S_0^{model\ stability} - S_t^{model\ stability}$	–	0.000041	0.000037	0.000042	0.000065	0.000089
$\sigma^{model\ stability}, \% \leq \max \sigma^{model}$	–	0.31	0.28	0.32	0.50	0.68
Approach 1	$\leq 4.52 \%$	–	satisfied	satisfied	satisfied	satisfied
Approach 2	$\leq 2.05 \%$	–	satisfied	satisfied	satisfied	satisfied

Table 2

The results of linearity verification of the secnidazole determination procedures by the method of HPLC/UV

Parameter	Values	Acceptability criterion			
		MCC		MS	
		Approach 1	Approach 2	Approach 1	Approach 2
<i>D</i> = 25-175 % (<i>g</i> = 7)					
b^{model}	0.981	–	–	–	–
s_b^{model}	0.017	–	–	–	–
a^{model}	–1.115	–	–	≤ 6.03 %	≤ 2.73 %
s_a^{model}	1.947	–	–	$a^{model} \leq 2.015 \cdot s_a^{model}$	
RSD_0^{model}	2.304	≤ 4.96 %	≤ 2.25 %	≤ 7.02 %	≤ 3.18 %
R_c^{model}	0.9992	≥ 0.9958	≥ 0.9991	≥ 0.9915	≥ 0.9983
<i>D</i> = 25-150 % (<i>g</i> = 6)					
b^{model}	0.964	–	–	–	–
s_b^{model}	0.020	–	–	–	–
a^{model}	0.006	–	–	≤ 6.03 %	≤ 2.73 %
s_a^{model}	1.990	–	–	$a^{model} \leq 2.132 \cdot s_a^{model}$	
RSD_0^{model}	2.138	≤ 4.69 %	≤ 2.12 %	≤ 6.63 %	≤ 3.00 %
R_c^{model}	0.9991	≥ 0.9950	≥ 0.9990	≥ 0.9899	≥ 0.9979
<i>D</i> = 25-125 % (<i>g</i> = 5)					
b^{model}	0.956	–	–	–	–
s_b^{model}	0.030	–	–	–	–
a^{model}	0.483	–	–	≤ 6.03 %	≤ 2.73 %
s_a^{model}	2.519	–	–	$a^{model} \leq 2.353 \cdot s_a^{model}$	
RSD_0^{model}	2.402	≤ 4.25 %	≤ 1.92 %	≤ 6.01 %	≤ 2.72 %
R_c^{model}	0.9985	≥ 0.9942	≥ 0.9988	≥ 0.9884	≥ 0.9976

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 LiClO₄ solution in 0.1 M HClO₄ solution): 2.2 ml of HClO₄ was measured by a 5.0 ml pipette into a 250.0 ml volumetric flask; the solution was diluted to the volume with *Solution 1*.

Eluent A: 10.0 ml of *Solution 2* was measured by the pipette into a 200.0 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 MiLiChrome® 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 °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. The volume of injection was 2 µL.

Table 3

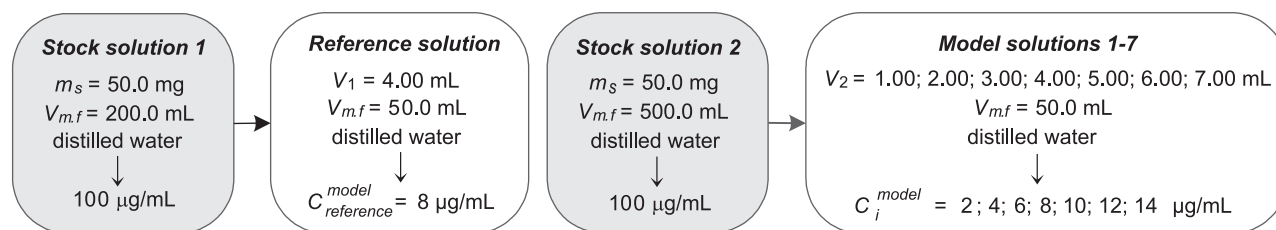
The results of accuracy and precision verification (MCC) of the secnidazole determination procedures by the method of HPLC/UV

Factual concentration of secnidazole in model solution ($C_{reference}^{model} = 8 \mu\text{g/mL}$)		Peak area S_i^{model}	Found in % to standard peak area $Y_i^{model}, \%$	Calculated concentration of secnidazole in model solution $X_{i,calc}^{model}, \%$			$RR_i^{model}, \%$		
$C_i^{model}, \mu\text{g/mL}$	$X_{i, fact}^{model}, \%$			25-175 %	25-150 %	25-125 %	25-175 %	25-150 %	25-125 %
2	25	0.003137	24.02	25.62	24.90	24.62	102.47	99.60	98.46
4	50	0.006261	47.94	50.00	49.71	49.64	100.01	99.43	99.28
6	75	0.009367	71.73	74.25	74.38	74.52	98.99	99.17	99.35
8	100	0.012997	99.52	102.58	103.20	103.59	102.58	103.20	103.59
10	125	0.015377	117.74	121.15	122.10	122.64	96.92	97.68	98.11
12	150	0.018979	145.32	149.26	150.71	–	99.51	100.47	–
14	175	0.022551	172.67	177.14	–	–	101.22	–	–
$S_{reference}^{model} = 0.013060$		$\overline{RR}^{model}, \%$					100.24	99.93	99.76
$\sigma^{model}, \% = 100 - \overline{RR}^{model} \leq \max \sigma^{model}$							0.24	0.07	0.24
				Approach 1	$\leq 4.52 \%$	satisfied	satisfied	satisfied	
				Approach 2	$\leq 2.05 \%$	satisfied	satisfied	satisfied	
$RSD_{RR}^{model}, \%$							2.02	1.84	2.20
$\Delta_{RR}^{model}, \% = RSD_{RR}^{model} \cdot t(95\%; g-1) \leq \max \Delta_{sample}^{model}$							3.93	3.72	4.70
				Approach 1	$\leq 10.00 \%$	satisfied	satisfied	satisfied	
				Approach 2	$\leq 4.52 \%$	satisfied	satisfied	unsatisfied	

Table 4

The results of accuracy and precision verification (MS) of the secnidazole determination procedures by the method HPLC/UV

Actual concentration of secnidazole in model solution ($C_{reference}^{model} = 8 \mu\text{g/mL}$)		Peak area S_i^{model}	Found in % to standard peak area $Y_i^{model}, \%$	$Z_i^{model}, \%$		
$C_i^{model}, \mu\text{g/mL}$	$X_{i, fact}^{model}, \%$			25-175 %	25-150 %	25-125 %
2	25	0.003137	24.02	96.07	96.07	96.07
4	50	0.006261	47.94	95.89	95.89	95.89
6	75	0.009367	71.73	95.63	95.63	95.63
8	100	0.012997	99.52	99.52	99.52	99.52
10	125	0.015377	117.74	94.19	94.19	94.19
12	150	0.018979	145.32	96.88	96.88	–
14	175	0.022551	172.67	98.67	–	–
$S_{reference}^{model} = 0.013060$		$\overline{Z}^{model}, \%$		96.69	96.36	96.26
$\sigma^{model}, \% = 100 - \overline{Z}^{model} \leq \max \sigma^{model}$				3.31	3.64	3.74
		Approach 1	$\leq 4.52 \%$	satisfied	satisfied	satisfied
		Approach 2	$\leq 2.05 \%$	unsatisfied	unsatisfied	unsatisfied
$RSD_Z^{model}, \%$				1.84	1.78	1.97
$\Delta_Z^{model}, \% = RSD_Z^{model} \cdot t(95\%; g-1) \leq \max \Delta_{As}^{model}$				3.58	3.58	4.19
		Approach 1	$\leq 14.14 \%$	satisfied	satisfied	satisfied
		Approach 2	$\leq 6.40 \%$	satisfied	satisfied	satisfied



Scheme 2. The preparation procedure for the reference and model solutions of secnidazole

Weighing was carried out using an AN100 digital analytical balance (AXIS, Ukraine) with $d = 0.0001$ g.

The glassware satisfied to ISO 648:2008 “Laboratory glassware – Single-volume pipettes”, ISO 1042:1998 “Laboratory glassware – One-mark volumetric flasks”, ISO 4788:2005 “Laboratory glassware – Graduated measuring cylinders”, ISO 385:2005 “Laboratory glassware – Burettes” and calibrated according to ISO 4787:2010 “Laboratory glassware – Volumetric instruments – Methods for testing of capacity and for use” and “Guidelines for calibration in analytical chemistry” [34] was used throughout this study.

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 50.0 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, more following the requirements to repeatability of peak areas S for

replicate injections offered by us [28] – the relative standard deviation of the mean RSD_{nom} calculated towards the nominal value of peak area S_{nom} should not exceed:

$$RSD_{nom} = \frac{s}{S_{nom}} \cdot 100 \% \leq \max RSD_{nom} = \frac{0.1 \cdot \max \Delta_{As} \cdot \sqrt{n}}{t(95\%; n-1)} = \begin{cases} 1.21 \% ; n = 3 \\ 1.74 \% ; n = 4 \\ 2.15 \% ; n = 5 \\ 2.49 \% ; n = 6 \end{cases} \quad (3)$$

where: S_{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.

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