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Research Article

Development and Validation of HPLC/UV-Spectrophotometric Procedures for Metronidazole Quantitative Determination

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ABSTRACT
Metronidazole is the most popular representative of antiprotozoal medicines from the group of 5-nitroimidazoles. Metronidazole blocks the enzymes of alcohol dehydrogenase and acetaldehyde dehydrogenase, therefore when its joint taking with alcohol it is observed the strong intoxication syndrome and fatal poisonings too. Therefore metronidazole can be a potential object of chemical toxicological investigations. The purpose of our paper is to develop HPLC/UV-procedure of metronidazole quantification with application of the system of HPLC-analyzer MiLiChrome® A-0230 implemented in practice of forensic medical laboratories in Russia and Ukraine and carry out step-by-step validation of the developed procedure. Chromatographic conditions: Eluent A (0.2 M $LiClO_4$ – 0.005 M $HClO_4$) and Eluent B (acetonitrile) were used as the mobile phase components; HPLC microcolumn Ø2×75 mm and ProntoSIL 120-5-C18 AQ, 5 μm were used as an analytical column; temperature was 40°C; flow rate was 100 μl/min; gradient elution mode was from 5% to 100% Eluent B for 40 min, then 100% Eluent B for 3 min; detection was performed at 277 nm. Retention time for metronidazole is 5.95 min. Since metronidazole is easy soluble and stable enough in the solutions of diluted alkalis 0.001 M sodium hydroxide solution has been proposed for preparation of the solutions in developing HPLC/UV-procedure of metronidazole quantification. Validation of the procedure has been carried out in the variants of the method of calibration curve and method of standard by such parameters as in process stability, linearity/calibration model, accuracy and precision within 3 different analytical runs using different batches of reagents and different glassware; experiments have been performed by three different analysts. New procedure of metronidazole quantitative determination by the method of HPLC/UV has been developed. Its validation has been carried out and acceptability for application has been shown.

Keywords: metronidazole, high-performance liquid chromatography, validation.

INTRODUCTION

The world pharmaceutical market is widely represented by medicines of the group of 5-nitroimidazole derivatives – metronidazole, ornidazole, tinidazole, etc.[1–4]. 5-nitroimidazoles are widely used for treatment of infectious diseases caused by Trichomonas, Lamblia, Leishmania, etc.[1–8]. The action mechanism of nitroimidazoles consists in biochemical reduction of 5-nitrogroup by intracellular transport proteins of anaerobes and protozoa. Reduced nitroimidazoles interact with DNA of microorganism cells and inhibit synthesis of their nucleic acids that leads to microorganism death[9–11].

Metronidazole is the most popular representative of this group of medicines. Administration of metronidazole is accompanied by a number of side effects such as unpleasant (metallic) taste and dry mouth, nausea, diarrhoea, abdominal pain, vomiting, headache, dizziness, depressive and convulsive reactions, skin itch, etc.[1–8,12]. Metronidazole blocks the enzymes of alcohol dehydrogenase and acetaldehyde dehydrogenase, therefore when joint taking the medicine with alcohol it is observed the strong intoxication syndrome manifested by intense vomiting, constant nausea, sharp headache, etc.; there is a «disulfiram-like response», when a person feels sudden blood rush to the head and upper body, feeling of difficulty in breathing, tinnitus, sharp reduction of blood pressure, tachycardia, and «death anxiety»[13–18]. Fatal poisonings with metronidazole have been recorded in the case of taking with alcohol[19].

Based on the mentioned above we can make the conclusion that metronidazole is a potential object of chemical toxicological investigations.

Chemically, metronidazole is 2-methyl-5-nitroimidazole-1-ethanol and has the structural formula as shown on Figure 1.

For 5-nitroimidazole determination the method of HPLC with different types of detection is widely used, it ensures high selectivity and sensitivity of analysis[20–27]. Chemical structure of metronidazole allows to use direct UV-spectrophotometry for its quantification, it is confirmed

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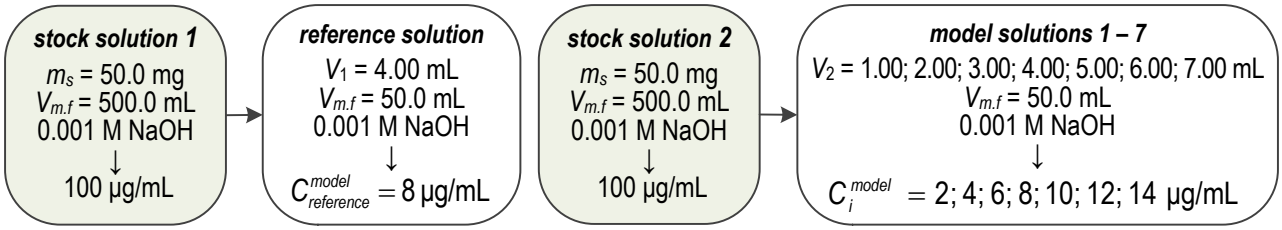

Scheme 1: The preparation procedure for reference and model solutions of metronidazole.

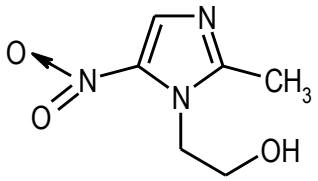

Figure 1: Chemical structure of metronidazole.

by us previously[28–29].

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

where S_{nom} – the mean peak area obtained when analysing the model solution 1. The mean values were used in further calculations.

The purpose of our paper is to develop HPLC/UV-procedure of metronidazole quantification with application of the system of HPLC-analyzer MiLiChrome® A-02[30] and carry out step-by-step validation of the developed procedure in the variants of the method of calibration curve (MCC) and method of standard (MS) to choose the optimal variant for further application in analytical toxicology.

MATERIALS AND METHODS

Reagents and chemicals

Metronidazole 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). Ethanol was of analytical grade.

Reference and model solutions (Scheme 1)

The stock solutions 1 and 2 (100 µg/mL) were prepared by dissolving 50.0 mg of metronidazole in 0.001 M sodium hydroxide solution and 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 0.001 M sodium hydroxide solution. The stock solution 2 was diluted with 0.001 M sodium hydroxide solution to prepare the model solutions 1 – 7 having concentrations of 2; 4; 6; 8; 10; 12 and 14 µg/mL respectively.

Mobile phase preparation

Eluent A (0.2 M $LiClO_4$ – 0.005 M $HClO_4$) and *Eluent B* (acetonitrile) were used as the mobile phase components. *Solution 1* and *Solution 2* were obtained for *Eluent A* preparation.

Solution 1 (4.1 M $LiClO_4$ aqueous solution): 330.00 g of $LiClO_4 \cdot 3H_2O$ were dissolved in 450 ml of bidistilled water while stirring and heating to 50°C, the solution obtained was cooled to ambient temperature and transferred to the measuring flask with the capacity of 500.0 ml, the solution was diluted to the volume with the same solvent and then filtered through the membrane filter Millex® HA Filter (0.45 µm pore size, 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 the pipette with the capacity of 5.0 ml into the measuring flask with the capacity of 250.0 ml, the solution was diluted to the volume with *Solution 1*.

Eluent A: 10.0 ml of *Solution 2* was measured by the pipette into the measuring flask with the capacity of 200.0 ml, the solution was diluted to the volume with bidistilled water.

Instrumentation and chromatographic conditions

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

Glassware satisfied 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»[31] was used throughout this study.

The HPLC/UV analyses were performed using high pressure liquid chromatograph MiLiChrome® A-02 (EcoNova, Russia) equipped with double syringe gradient pump, autosampler (sample volume is 0 – 99 µl), column oven (35 – 90°C) and double-beam multiwave UV-spectrophotometer as a detector. Analitika-Chrom® software (Analitika SPF, Ukraine) was used for integration and processing of chromatograms. HPLC microcolumn of Ø2×75 mm dimension and reversed phase ProntoSIL 120-5-C18 AQ, 5 µm (BISCHOFF Analysentechnik und -geräte GmbH, Germany) were used as an analytical column. All analysis was carried out at 40°C and flow rate of 100 µl/min. The mobile phase was run in gradient elution mode, namely from 5% to 100% *Eluent B* for 40 min, then 100% *Eluent B* for 3 min. Detection was performed at 247 nm. The volume of injection was 2 µL.

When experiments carrying out each solution (excepting in process stability studying) was chromatographed 3 times or, as required, more following the requirements to

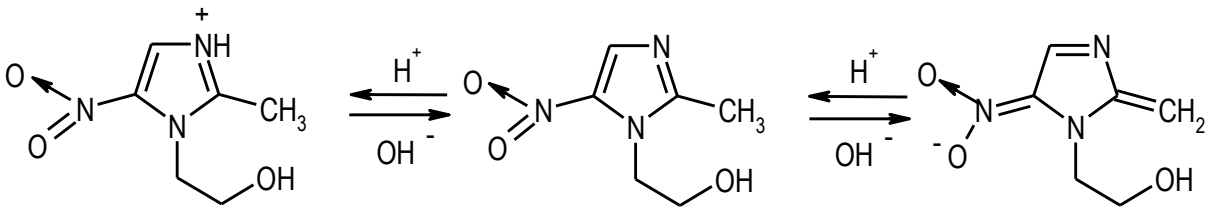

Figure 2: Possible transformations in the metronidazole solutions when changing the medium pH.

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

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

$$\max\Delta_{cal}^{model} = \max\Delta_{sample}^{model} = \frac{\max\Delta_{As}^{model}}{\sqrt{2}} = 0.707 \cdot \max\Delta_{As}^{model} = 0.707 \cdot 14.14\% = 10.00\%;$$

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

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

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

$$\max\Delta_{cal}^{model} = \max\Delta_{sample}^{model} = \frac{\max\Delta_{As}^{model}}{\sqrt{2}} = 0.707 \cdot \max\Delta_{As}^{model} = 0.707 \cdot 6.40\% = 4.52\%;$$

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

repeatability of peaks areas *S* for replicate injections offered[32] – the relative standard deviation of the mean RSD_{nom} calculated towards the nominal value of peak area S_{nom} should not exceed:

RESULTS AND DISCUSSION

HPLC is used to analyse metronidazole in pharmaceuticals and biological liquids widely enough[20–22,24–26]. The main disadvantage of the present procedures is their application exclusively for metronidazole or mixture of 5-nitroimidazoles quantification; chromatographic conditions are specially chosen to analyse only this group of medicines. It is usual situation for pharmacokinetic studies, but in forensic toxicology it is impossible to use individual procedures for each analyte, it is necessary to use unified technics of sample preparation and unified screening chromatographic conditions, so called HPLC-analyzer system.

HPLC-analyzer MiLiChrome® A-02 is implemented in practice of forensic medical laboratories in Russia and Ukraine[30]. Previously[33] the specificity of chromatographic conditions of HPLC-analyzer MiLiChrome® A-02 application for metronidazole determination has been confirmed in relation to other medicines of the group of 5-nitroimidazoles (secnidazole, tinidazole, ornidazole and nimorazole). Retention time for metronidazole is 5.95 min, unlike for secnidazole (8.16 min), tinidazole (9.13 min), ornidazole (10.18 min) and nimorazole (14.12 min)[33].

We have previously[29] shown the possibility of application of direct UV-spectrophotometry for metronidazole quantitative determination using three solvents – 0.1 M hydrochloric acid solution, 96% ethanol and 0.1 M sodium hydroxide solution (analytical wavelengths λ_{max} are 277 nm, 310 nm and 319 nm respectively). All solvents provide sufficient stability of the medicines[29].

Since metronidazole is easy soluble and stable enough[29] in the solutions of diluted alkalis 0.001 M sodium hydroxide solution has been proposed by us for preparation of the reference and model solutions in developing HPLC/UV-procedure of metronidazole quantification. Under these conditions pH of the solutions is satisfied to the requirements to the samples injected to the HPLC-analyzer MiLiChrome® A-02[30] (pH of mobile phase is more than 2.3), since it does not affect pH of eluent. In this case detection should be carried out at 277 nm, which corresponds to the absorption maximum of acid form of metronidazole (Figure 2).

To prove the possibility of the proposed procedure application in further analysis its validation has been carried out in the variants of the method of calibration curve[32,34–37] and method of standard[32,38].

Such validation parameters as in process stability, linearity/calibration model, accuracy and precision (repeatability) have been estimated by model solutions.

Method validation by model solutions according to Scheme 2 suggested according the requirements[32] allows to assess the suitability of the actual analytical procedure for further work.

The validation provides application of the normalized coordinates, i. e. transition from the equation

in process stability

analysis of the reference solution in 0, 1, 12, 24 and 48 h

$$C_{reference}^{model} \cong S_{reference}^{model} \cong 100\%; \quad S_t^{model\ stability}; \quad \delta^{model\ stability} = \frac{\left|S_{reference}^{model} - S_t^{model\ stability}\right|}{S_{reference}^{model}} \cdot 100\%$$

Approach 1: $\Delta_{As}^{model} = \Delta_{sample\ preparation}$

$\delta^{model\ stability} \le \max\delta^{model} = 4.52\%$

Approach 2: $\Delta_{As}^{model} \le 0.32 \cdot \Delta_{As}$

$\delta^{model\ stability} \le \max\delta^{model} = 2.05\%$

analysis of the model solutions 1 – 7 (*1 run – 1 day*)

$$C_i^{model} \cong S_i^{model} \cong 25, 50, 75, 100, 125, 150, 175\%; \quad X_{i,fact}^{model} = \frac{C_i^{model}}{C_{reference}^{model}} \cdot 100\%; \quad Y_i^{model} = \frac{S_i^{model}}{S_{reference}^{model}} \cdot 100\%$$

linearity/calibration model

$$Y^{model} = a + b \cdot X^{model} \rightarrow a^{model}; s_a^{model}; b^{model}; s_b^{model}; RSD_0^{model}; R_c^{model}$$

MCC

Approach 1: $\Delta_{As}^{model} = \Delta_{sample\ preparation}$

$D = 25-175\%,\ g = 7 \rightarrow RSD_0^{model} \le 4.96\%,\ R_c^{model} \ge 0.9958$

$D = 25-150\%,\ g = 6 \rightarrow RSD_0^{model} \le 4.69\%,\ R_c^{model} \ge 0.9950$

$D = 25-125\%,\ g = 5 \rightarrow RSD_0^{model} \le 4.25\%,\ R_c^{model} \ge 0.9942$

Approach 2: $\Delta_{As}^{model} \le 0.32 \cdot \Delta_{As}$

$D = 25-175\%,\ g = 7 \rightarrow RSD_0^{model} \le 2.25\%,\ R_c^{model} \ge 0.9991$

$D = 25-150\%,\ g = 6 \rightarrow RSD_0^{model} \le 2.12\%,\ R_c^{model} \ge 0.9990$

$D = 25-125\%,\ g = 5 \rightarrow RSD_0^{model} \le 1.92\%,\ R_c^{model} \ge 0.9988$

MS

Approach 1: $\Delta_{As}^{model} = \Delta_{sample\ preparation}$

$a^{model}: 1) \le t(95\%; g-2) \cdot s_a^{model};\ 2) \le 6.03\%$

$D = 25-175\%,\ g = 7 \rightarrow RSD_0^{model} \le 7.02\%,\ R_c^{model} \ge 0.9915$

$D = 25-150\%,\ g = 6 \rightarrow RSD_0^{model} \le 6.63\%,\ R_c^{model} \ge 0.9899$

$D = 25-125\%,\ g = 5 \rightarrow RSD_0^{model} \le 6.01\%,\ R_c^{model} \ge 0.9884$

Approach 2: $\Delta_{As}^{model} \le 0.32 \cdot \Delta_{As}$

$a^{model}: 1) \le t(95\%; g-2) \cdot s_a^{model};\ 2) \le 2.73\%$

$D = 25-175\%,\ g = 7 \rightarrow RSD_0^{model} \le 3.18\%,\ R_c^{model} \ge 0.9983$

$D = 25-150\%,\ g = 6 \rightarrow RSD_0^{model} \le 3.00\%,\ R_c^{model} \ge 0.9979$

$D = 25-125\%,\ g = 5 \rightarrow RSD_0^{model} \le 2.72\%,\ R_c^{model} \ge 0.9976$

accuracy and repeatability

MCC

$$X_{i,calc}^{model} = \frac{Y_i^{model} - a^{model}}{b^{model}}; \quad RR_i^{model} = \frac{X_{i,calc}^{model}}{X_{i,fact}^{model}} \cdot 100\%; \quad \Delta_{RR}^{model} = t(95\%; g-1) \cdot RSD_{RR}^{model}; \quad \delta^{model} = \left|100 - \overline{RR}^{model}\right|$$

Approach 1: $\Delta_{As}^{model} = \Delta_{sample\ preparation}$

$\Delta_{RR}^{model} \le \max\Delta_{sample}^{model} = 10.00\%$

$\delta^{model} \le \max\delta^{model} = 4.52\%$

Approach 2: $\Delta_{As}^{model} \le 0.32 \cdot \Delta_{As}$

$\Delta_{RR}^{model} \le \max\Delta_{sample}^{model} = 4.52\%$

$\delta^{model} \le \max\delta^{model} = 2.05\%$

MS

$$Z_i^{model} = \frac{Y_i^{model}}{X_{i,fact}^{model}} \cdot 100\%; \quad \Delta_Z^{model} = t(95\%; g-1) \cdot RSD_Z^{model}; \quad \delta^{model} = \left|100 - \overline{Z}^{model}\right|$$

Approach 1: $\Delta_{As}^{model} = \Delta_{sample\ preparation}$

$\Delta_Z^{model} \le \max\Delta_{As}^{model} = 14.14\%$

$\delta^{model} \le \max\delta^{model} = 4.52\%$

Approach 2: $\Delta_{As}^{model} \le 0.32 \cdot \Delta_{As}$

$\Delta_Z^{model} \le \max\Delta_{As}^{model} = 6.40\%$

$\delta^{model} \le \max\delta^{model} = 2.05\%$

Scheme 2: The validation stages of HPLC/UV-procedure for metronidazole determination.

For normalization of the obtained experimental data the

Table 1: The results of in process stability verification for metronidazole in model solutions.

Parameter	Values					
	0 h	1 h	12 h	24 h	36 h	48 h
$S^{model\ stability}$	0.016873	0.016916	0.017016	0.017026	0.016883	0.016852
$S_0^{model\ stability} - S_t^{model\ stability}$	–	0.000043	0.000143	0.000153	0.000010	0.000021
$\delta^{model\ stability}, \% \le \max \delta^{model}$	–	0.25	0.85	0.91	0.06	0.12
Approach 1 $\le 4.52\%$	–	satisfied	satisfied	satisfied	satisfied	satisfied
Approach 2 $\le 2.05\%$	–	satisfied	satisfied	satisfied	satisfied	satisfied

$A_i = b_1 \cdot C_i + a_1$ to the equation $Y_i = b_2 \cdot X_i + a_2$,
that allows to calculate the validation characteristics, which do not depend on the analyte and features of the method of analysis[39,40].

The metronidazole concentration in the model solution for the point of 100% in the normalized coordinates $C_{100\%}^{model}$ has been chosen as the concentration provided the «signal/noise» ratio at the level of 40[32].

reference solution with the analyte concentration of $C_{reference}^{model} = C_{100\%}^{model}$ is used.

The analytical ranges *D* of the method application are 25 – 125%, 25 – 150% and 25 – 175%; the number of concentration levels *g* equals 5, 6 or 7 respectively in constant increments of 25%.

Acceptability criteria for validation parameters have been formed on the basis of systematic application of "insignificance concept"[39,40] and proceeding from the value of

Table 2: The results of linearity verification of metronidazole determination procedure by the method of HPLC/UV.

Parameter	Values	Acceptability criterion			
		MCC		*MS*	
		Approach 1	*Approach 2*	*Approach 1*	*Approach 2*
D = 25 – 175% (*g* = 7)					
b^{model}	1.010	–	–	–	–
s_b^{model}	0.011	–	–	–	–
a^{model}	–1.058	–	–	$\le 6.03\%$	$\le 2.73\%$
s_a^{model}	1.203	–	–	$a^{model} \le 2.015 \cdot s_a^{model}$	
RSD_0^{model}	1.423	$\le 4.96\%$	$\le 2.25\%$	$\le 7.02\%$	$\le 3.18\%$
R_c^{model}	0.9997	≥ 0.9958	≥ 0.9991	≥ 0.9915	≥ 0.9983
D = 25 – 150% (*g* = 6)					
b^{model}	0.998	–	–	–	–
s_b^{model}	0.012	–	–	–	–
a^{model}	–0.262	–	–	$\le 6.03\%$	$\le 2.73\%$
s_a^{model}	1.136	–	–	$a^{model} \le 2.132 \cdot s_a^{model}$	
RSD_0^{model}	1.221	$\le 4.69\%$	$\le 2.12\%$	$\le 6.63\%$	$\le 3.00\%$
R_c^{model}	0.9997	≥ 0.9950	≥ 0.9990	≥ 0.9899	≥ 0.9979
D = 25 – 125% (*g* = 5)					
b^{model}	1.016	–	–	–	–
s_b^{model}	0.007	–	–	–	–
a^{model}	–1.350	–	–	$\le 6.03\%$	$\le 2.73\%$
s_a^{model}	0.568	–	–	$a^{model} \le 2.353 \cdot s_a^{model}$	
RSD_0^{model}	0.542	$\le 4.25\%$	$\le 1.92\%$	$\le 6.01\%$	$\le 2.72\%$
R_c^{model}	0.9999	≥ 0.9942	≥ 0.9988	≥ 0.9884	≥ 0.9976

extreme uncertainty Δ_{As}, which equals 20% for the method in analytical toxicology[41,42].

In the *MCC* acceptability criteria for linear dependence and precision have been 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 have been calculated proceeding from two approaches:

In process stability of metronidazole 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 $\delta^{model\,stability}$ was calculated and assessed (Table 1). *In process stability* of metronidazole in model solutions is satisfied the acceptability criteria for all periods of time both for *Approach 1* and *Approach 2*.

To determine *linearity/calibration model* the model solutions 1 – 7 were analysed within 1 run, correlation coefficient R_c^{model}, rest standard deviation RSD_0^{model} and also absolute term a^{model} (if it is necessary) were calculated and assessed (Table 2).

To estimate *precision (repeatability) and accuracy:*

MCC: the model solutions 1 – 7 concentrations 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 δ^{model} respectively (Table 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 δ^{model} respectively (Table 4).

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

Validation of the procedure has been carried out within 3 different analytical runs using different batches of reagents and different glassware; experiments have been performed by three different analysts. The results obtained within one analytical run are presented in Tables 1 – 4, but results of other analytical runs are at the same range of values.

The total results of validation allow to point to the conclusion about acceptable *linearity*, *accuracy* and *precision* of HPLC/UV-procedure of metronidazole quantitative determination in the variant of the MCC and MS for all ranges of the method application and for both ap

Table 3: The results of accuracy and precision verification (MCC) of metronidazole determination procedure by the method of HPLC/UV.

Factual concentration of metronidazole in model solution ($C_{reference}^{model} = 8$ μg/mL)		Peak area S_i^{model}	Found in % to standard peak area Y_i^{model},%	Calculated concentration of metronidazole in model solution $X_{i,calc}^{model}$,%			RR_i^{model},%		
C_i^{model}, μg/mL	$X_{i,fact}^{model}$,%			25 – 175%	25 – 150%	25 – 125%	25 – 175%	25 – 150%	25 – 125%
2	25	0,004146	24.23	25.05	24.55	25.18	100.21	98.22	100.70
4	50	0,008436	49.31	49.89	49.69	49.85	99.78	99.38	99.70
6	75	0,012850	75.11	75.45	75.56	75.24	100.60	100.74	100.32
8	100	0,017029	99.54	99.64	100.04	99.27	99.64	100.04	99.27
10	125	0,021582	126.15	126.01	126.72	125.46	100.81	101.37	100.37
12	150	0,025290	147.82	147.47	148.44	–	98.32	98.96	–
14	175	0,030300	177.11	176.48	–	–	100.85	–	–
$S_{reference}^{model} = 0.017108$			$\overline{RR}^{model}$,%				100.03	99.79	100.07
$\delta^{model},\% = \left\|100 - \overline{RR}^{model}\right\| \le \max\delta^{model}$							0.03	0.21	0.07
				Approach 1	≤ 4.52%		satisfied	satisfied	satisfied
				Approach 2	≤ 2.05%		satisfied	satisfied	satisfied
$RSD_{RR}^{model},\%$							0.89	1.17	0.57
$\Delta_{RR}^{model},\% = RSD_{RR}^{model} \cdot t(95\%; g-1) \le \max\Delta_{sample}^{model}$							1.73	2.35	1.23
				Approach 1	≤ 10.00%		satisfied	satisfied	satisfied
				Approach 2	≤ 4.52%		satisfied	satisfied	satisfied

Table 4: The results of accuracy and precision verification (MS) of metronidazole determination procedure by the method of HPLC/UV.

Factual concentration of metronidazole in model solution ($C_{reference}^{model} = 8$ μg/mL)		Peak area S_i^{model}	Found in % to standard peak area Y_i^{model},%	Z_i^{model},%		
C_i^{model}, μg/mL	$X_{i,fact}^{model}$,%			25 – 175%	25 – 150%	25 – 125%
2	25	0.004146	24.23	96.94	96.94	96.94
4	50	0.008436	49.31	98.62	98.62	98.62
6	75	0.012850	75.11	100.15	100.15	100.15
8	100	0.017029	99.54	99.54	99.54	99.54
10	125	0.021582	126.15	100.92	100.92	100.92
12	150	0.025290	147.82	98.55	98.55	–
14	175	0.030300	177.11	101.21	–	–
$S_{reference}^{model} = 0.017108$		$\bar{Z}^{model}$,%		99.42	99.12	99.23
$\delta^{model},\% = \left\|100 - \bar{Z}^{model}\right\| \le \max\delta^{model}$				0.58	0.88	0.77
		Approach 1	≤ 4.52%	satisfied	satisfied	satisfied
		Approach 2	≤ 2.05%	satisfied	satisfied	satisfied
$RSD_Z^{model},\%$				1.50	1.40	1.54
$\Delta_Z^{model},\% = RSD_Z^{model} \cdot t(95\%; g-1) \le \max\Delta_{As}^{model}$				2.92	2.83	3.27
		Approach 1	≤ 14.14%	satisfied	satisfied	satisfied
		Approach 2	≤ 6.40%	satisfied	satisfied	satisfied

proaches 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 of biological liquids analysis for metronidazole 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. MCC, undoubtedly, allows to take into account and partially level the influence of matrix background absorption on the results of determination, but proves its value only in the case of routine analyses carrying out. In forensic toxicological analysis we often meet with one-time examinations, and various biological fluids, organs and tissues are sent for the examinations, that is it is necessary to determine analyte quantitatively in some various biological objects, and the necessity of carrying out such determinations can arise rarely enough. In such situation plotting the calibration curve for each matrix demands quite nonrational investment of time, and to the moment of obtaining the results of analysis they can become irrelevant. That makes the variant of MS more optimal for analysis.

CONCLUSIONS

New procedure of metronidazole 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 calibration curve and method of standard has been carried out and acceptability for application has been shown.

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