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Development and validation of HPLC/UV-procedures for quantification of metronidazole in blood and urine

Metronidazole belongs to the group of antiprotozoal medicines and widely used for the treatment of infectious diseases; the medicine has a number of side effects manifested by usual symptoms of acute intoxication, especially when interacting with other drugs and alcohol.

Aim. To apply the system of MiLiChrome® A-02 HPLC-analyzer widely used in the Ukrainian laboratories of forensic toxicology for the metronidazole quantitative determination in biological fluids and carry out validation of the procedures developed.

Materials and methods. The sample preparation of blood and urine was carried out by extraction with acetonitrile and 2-propanol followed by separation of the organic layer under the conditions of the aqueous phase saturation with ammonium sulfate. Previously blood and urine were treated with acids. Isolation was carried out in the strong acid, neutral and weak alkaline medium.

Results and discussion. To find the optimal conditions of the sample preparation such validation parameters as specificity/selectivity and recovery were determined. The results of the blank samples analysis were acceptable for all variants of the sample preparation procedures. Recovery values were reproducible for all procedures of analysis studied, but efficacy of metronidazole isolation was variable – from 85 % to 97 %. The results of verification of metronidazole stability showed the necessity to carry out all measurements within 12 hours after obtaining the solutions to be analyzed. The results of determination of linearity, accuracy and precision were the evidence of acceptable systematic and random errors of the HPLC/UV-procedures studied in the variant of the method of calibration curve, method of standard and method of additions.

Conclusions. The set of HPLC/UV-procedures for the metronidazole quantitative determination in blood and urine has been developed. Validation of the procedures developed has been carried out.

***Key words*:** metronidazole; high-performance liquid chromatography; blood; urine; sample preparation; validation; method of calibration curve; method of standard; method of additions

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Розробка і валідація ВЕРХ/УФ-методик кількісного визначення метронідазолу в крові та сечі

Метронідазол належить до групи антипротозойних засобів і широко застосовується для лікування інфекційних захворювань; препарат має ряд побічних ефектів, що виявляються звичайними симптомами гострої інтоксикації, особливо при взаємодії з іншими препаратами і алкоголем.

Мета. Застосувати систему ВЕРХ-аналізатора MiLiChrome® A-02, що широко використовується в українських судово-токсикологічних лабораторіях, для кількісного визначення метронідазолу в біологічних рідинах і провести валідацію розроблених методик.

Матеріали та методи. Пробопідготовку крові і сечі проводили шляхом екстракції ацетонітрилом і ізопропанолом з подальшим відділенням органічного шару в умовах насичення водної фази амонію сульфатом. Попередньо кров і сечу обробляли кислотами. Ізолювання проводили в сильнокислому, нейтральному і слабколужному середовищі.

Результати та їх обговорення. Для підбору оптимальних умов пробопідготовки були визначені такі валідаційні параметри як специфічність/селективність і ступінь ізолювання. Результати аналізу *blank*-проб прийнятні для всіх варіантів процедур пробопідготовки. Значення ступеня ізолювання відтворювані для всіх вивчених процедур аналізу, але ефективність виділення метронідазолу різна – від 85 % до 97 %. Результати перевірки стабільності метронідазолу показали необхідність проведення всіх вимірювань впродовж 12 годин після отримання аналізованих розчинів. Результати визначення лінійності, правильності та прецизійності свідчать про допустимість систематичних і випадкових помилок досліджених ВЕРХ/УФ-методик у варіанті методу калібрувального графіка, методу стандарту та методу добавок.

Висновки. Розроблено комплекс ВЕРХ-методик для кількісного визначення метронідазолу в крові і сечі. Проведено валідацію розроблених методик.

***Ключові слова*:** метронідазол; високоефективна рідинна хроматографія; кров; сеча; пробопідготовка; валідація; метод калібрувального графіка; метод стандарту; метод добавок

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Разработка и валидация ВЭЖХ/УФ-методик количественного определения метронидазола в крови и моче

Метронидазол относится к группе антипротозойных препаратов и широко применяется для лечения инфекционных заболеваний; препарат имеет ряд побочных эффектов, проявляющихся обычными симптомами острой интоксикации, особенно при взаимодействии с другими препаратами и алкоголем.

Цель. Применить систему ВЭЖХ-анализатора MiLiChrome® A-02, широко используемую в украинских судебно-токсикологических лабораториях, для количественного определения метронидазола в биологических жидкостях и провести валидацию разработанных методик.

Материалы и методы. Пробоподготовку крови и мочи проводили путем экстракции ацетонитрилом и изопропанолом с последующим отделением органического слоя в условиях насыщения водной фазы аммония сульфатом. Предварительно кровь и мочу обрабатывали кислотами. Изолирование проводили в сильнокислой, нейтральной и слабощелочной среде.

Результаты и их обсуждение. Для подбора оптимальных условий пробоподготовки были определены такие валидационные параметры, как специфичность/селективность и степень извлечения. Результаты анализа *blank*-проб приемлемы для всех вариантов процедур пробоподготовки. Значения степени извлечения воспроизводимы для всех изученных процедур анализа, но эффективность выделения метронидазола различна – от 85 % до 97 %. Результаты проверки стабильности метронидазола показали необходимость проведения всех измерений в течение 12 часов после получения анализируемых растворов. Результаты определения линейности, правильности и прецизионности свидетельствуют о допустимости систематических и случайных ошибок изученных ВЭЖХ/УФ-методик в варианте метода калибровочного графика, метода стандарта и метода добавок.

Выводы. Разработан комплекс ВЭЖХ-методик для количественного определения метронидазола в крови и моче. Проведена валидация разработанных методик.

***Ключевые слова*:** метронидазол; высокоэффективная жидкостная хроматография; кровь; моча; пробоподготовка; валидация; метод калибровочного графика; метод стандарта; метод добавок

At the Analytical Chemistry Department of the National University of Pharmacy (Kharkiv, Ukraine) the research in the field of creating the unified standardized validation procedures for methods of the analyte quantification in different biological matrices for application in forensic toxicology is carried out [1-9]. The main purposes of such unified standardized validation procedures are to provide development of the method with the optimal pre-specified parameters and acceptance of the method as legitimate.

As a part of the research mentioned the experiment for development of the method for metronidazole quantification in blood and urine using HPLC was performed.

Metronidazole belongs to the group of antiprotozoal medicines and is widely used for the treatment of infectious diseases caused by *Trichomonas*, *Lamblia*, *Leishmania*, etc., as well as in the treatment of peptic ulcer associated with *Helicobacter pylori* [10-12]. The medicine has a number of side effects manifested by usual symptoms of acute intoxication (giddiness, nausea, vomiting), especially when interacting with other drugs and alcohol [13-14]. And the case of interaction with alcohol may be lethal for a patient even when the therapeutic dose is taken [15]. According to unpublished data metronidazole is identified sometimes in human biological matrices in toxicological examinations in Ukraine.

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

HPLC is used to analyze metronidazole in pharmaceuticals and biological fluids rather widely [16-18]. The main disadvantage of the present procedures is their application exclusively for metronidazole quantification; both chromatographic conditions and sample preparations are specially chosen to analyze metronidazole. 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 methods of sample preparation and unified screening chromatographic conditions, so-called HPLC-analyzer system.

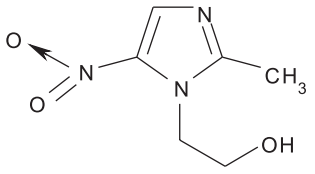


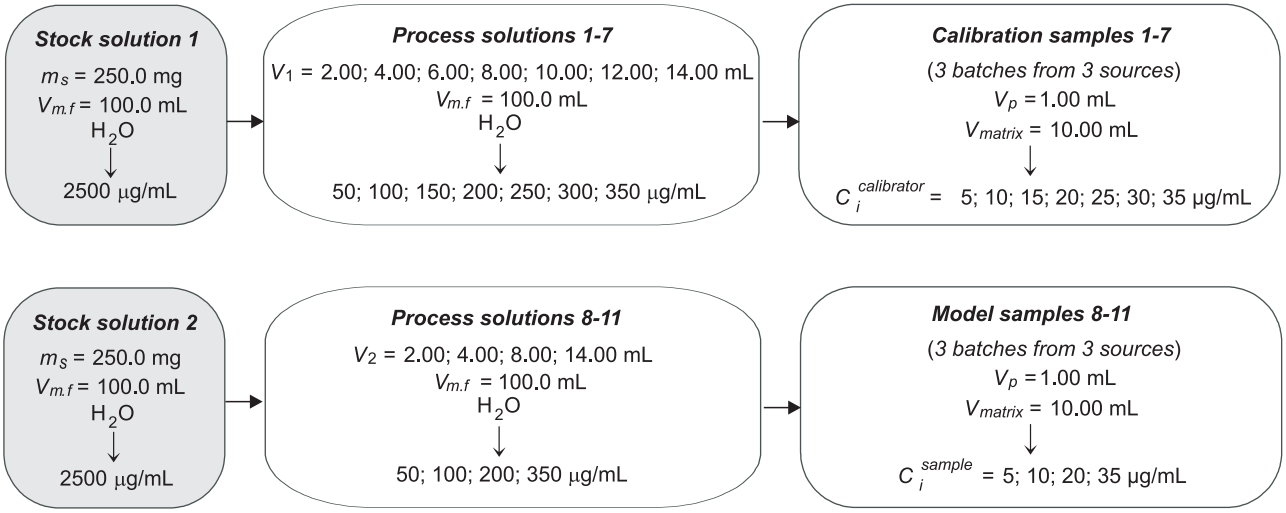
Fig. Chemical structure of metronidazole

Thus, this research was conducted to develop some HPLC-procedures of metronidazole quantification in blood and urine using different traditional methods of sample preparation [19-20] and approved in Ukraine for application in forensic toxicology. The HPLC-analysis was carried out using the system of MiLiChrome® A-02 HPLC-analyzer [21] implemented in practice of forensic medical laboratories in Russia and Ukraine. The step-by-step validation of the procedures developed was performed according to the approaches offered by us [1-9] to choose the optimal variants of sample preparation provided high accuracy and precision, enough sensitivity and specificity, etc. Another aim of our experiment was to accumulate the experience of application of the standardized validation procedures offered for the method development.

Materials and methods

Reagents and chemicals

Metronidazole was of pharmacopoeial purity and obtained from the pharmaceutical company "Zdorovie" Ltd. Acetonitrile (99.8 %, anhydrous), hydrochloric acid (≥ 37 %, puriss. p.a., ACS reagent, fuming), trichloroacetic acid (≥ 99.0 %, ACS reagent), chloroform (≥ 99 %, anhydrous, contains 0.5-1.0 % of ethanol as a stabilizer), methanol (≥ 99.8 %, puriss. p.a., ACS reagent), 2-propanol (LC-MS CHROMASOLV®), ammonium hydroxide solution (≥ 25 % NH_3 in H_2O, puriss. p.a. plus) were purchased from Sigma-Aldrich Co. LLC (USA). All other reagents (sodium sulfate anhydrous, ammonium sulfate) were of analytical grade. The chromatographic plates Sorbfil® PTLC-PH (silica gel STC-1HP,



Scheme 1. The preparation procedure for calibration and model samples of metronidazole for MCC and MS

PETP, silica sol, 8 ÷ 12 μm fraction, the layer thickness of 100 μm) were purchased from IMID LLC (Russia).

Calibration and model samples

Blank-samples were prepared by spiking 5 samples (10.00 mL) of the corresponding matrix (blood or urine) obtained from different sources with 1.00 mL of distilled water.

The method of calibration curve (MCC) and the method of standard (MS) – Scheme 1

The stock solutions 1 and 2 (2500 μg/mL) were prepared by dissolving 250.0 mg of metronidazole in distilled water, and the solutions were diluted to 100.0 mL with the same solvent. The stock solutions 1 and 2 were diluted with distilled water to prepare:

- the process solutions 1-7 having the concentrations of 50; 100; 150; 200; 250; 300; 350 μg/mL, respectively;
- the process solutions 8-11 having the concentrations of 50; 100; 200 and 350 μg/mL, respectively.

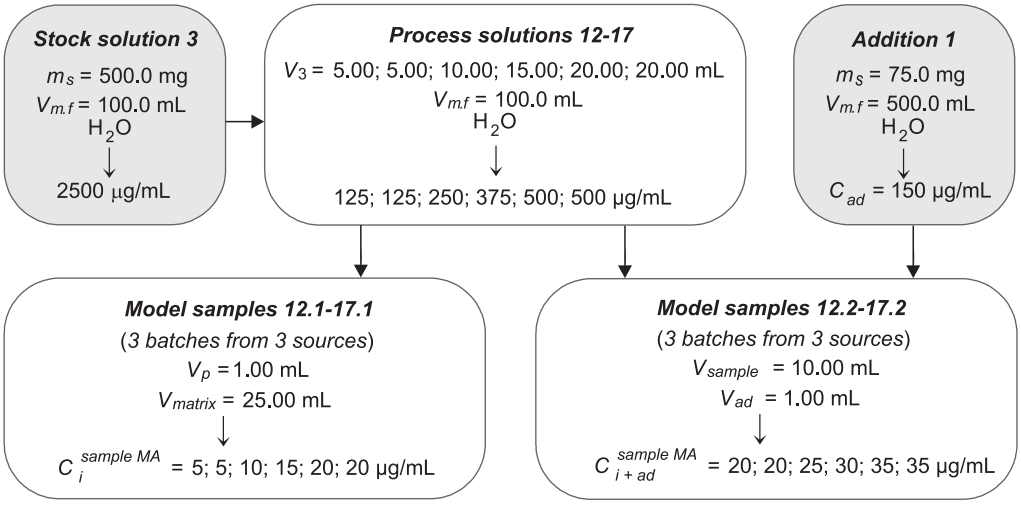
Three batches (in 11 samples each) of the corresponding matrix (blood or urine) obtained from three different sources were used to prepare the calibration samples 1-7 and the model samples 8-11 by spiking 10.00 mL of the matrix with 1 mL of the process solutions 1-11, respectively.

After spiking all samples were vortexed for 1 hour and stored for 24 hours at ambient temperature before the sample processing.

The method of additions (MA) – Scheme 2

The stock solution 3 (2500 μg/mL) was prepared by dissolving 250.0 mg of metronidazole in distilled water, and the solution was diluted to 100.0 mL with the same solvent. The of addition solution 1 (150 μg/mL) was prepared by dissolving 75.0 mg of metronidazole in distilled water, and the solution was diluted to 500.0 mL with the same solvent. The stock solution 3 was diluted with distilled water to prepare the process solutions 12-17 having the concentrations of 125; 125; 250; 375; 500; 500 μg/mL, respectively.

Three batches (in 6 samples each) of the corresponding matrix (blood or urine) obtained from three different sources were used to prepare the model samples 12.1-17.1 by spiking 25.00 mL of the matrix with 1.00 ml of the process solutions 12-17, respectively.



Scheme 2. The preparation procedure for model samples of metronidazole for MA

After spiking all samples were vortexed for 1 hour and stored for 24 hours at ambient temperature before the sample processing.

To prepare the model samples 12.2-17.2 10.00 mL of the model samples 12.1-17.1 were spiked with 1.00 mL of the solution of addition 1 directly before the sample processing.

Reference and model solutions

The stock solutions 4 and 5 (100 μg/mL) were prepared by dissolving 50.0 mg of metronidazole in 0.01 M hydrochloric acid solution, and the solutions were diluted to 500.0 mL with the same solvent. The reference solution (8 μg/mL) was prepared by diluting 8.00 mL of the stock solution 4 to 100.0 mL with 0.01 M hydrochloric acid solution. The stock solution 5 was diluted with 0.01 M hydrochloric acid solution to prepare the model solutions 1-4 having the concentrations of 2; 4; 8 and 14 μg/mL, respectively.

Blood and urine sample preparation for the metronidazole determination – Scheme 3

Blood: 10.00 mL of blood was diluted with 20.00 mL of distilled water and processed with 10.00 mL of 10 % trichloroacetic acid aqueous solution. The mixture was vortexed for 1 hour, then centrifuged for 5 minutes at 5000 rpm.

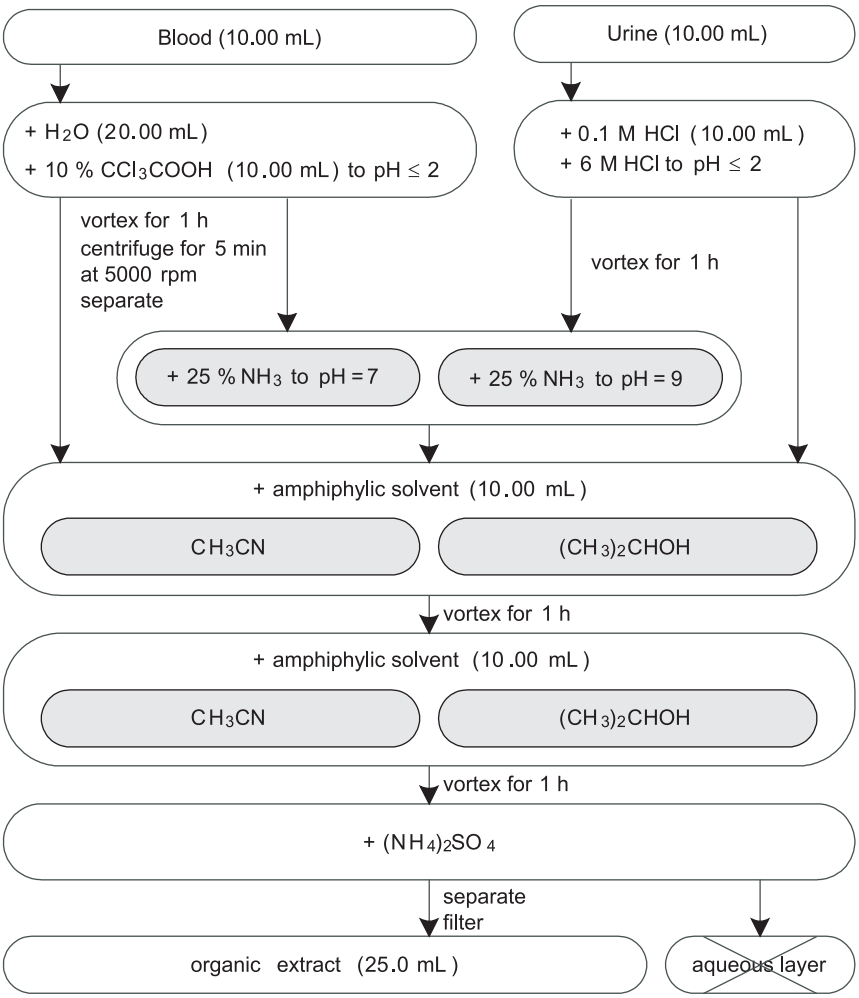
Urine: 10.00 mL of urine was processed with 10.00 mL of 0.1 M hydrochloric acid solution and then acidified with 6 M hydrochloric acid solution to pH ≤ 2.

The next stages were the same for blood and urine.

Procedure 1-1: The supernatant was processed twice with 10.00 mL of acetonitrile and vortexed for 1 hour each time. After adding 2 g of ammonium sulfate the mixture was filtered through the paper filter (wetted with acetonitrile) into a separating funnel and salted-out by adding ammonium sulfate till its dissolution stops. The top organic layer was separated, filtered through the paper filter with 1 g of anhydrous sodium sulfate into a 25.0 mL measuring flask, and diluted to the volume with acetonitrile.

Procedure 1-2: The supernatant was neutralized with 25 % ammonium hydroxide solution to pH = 7. The next stages were as for **Procedure 1-1**.

Procedure 1-3: The supernatant was alkalified with 25 % ammonium hydroxide solution to pH = 9. The next stages were as for **Procedure 1-1**.



Scheme 3. The main stages of blood and urine sample preparation for metronidazole quantification

Procedure 2-1, 2-2 and 2-3: All stages were as for **Procedure 1-1, 2-1** and **3-1**, respectively, but 2-propanol was used instead of acetonitrile.

TLC-purification: 10.00 mL of the organic extract obtained were evaporated at 80 °C to complete removal of the organic layer; a dry residue was dissolved in ~0.5 mL of chloroform and applied quantitatively on the start line of the chromatographic plate in the form of a band of 2 cm in width. 10 μL of metronidazole standard ethanol solution (1 mg/mL) were applied in the point ("testifier") near the band. The plate was eluted in chloroform twice and then dried out using the mixture of chloroform and methanol (90 : 10) as a mobile phase; the "testifier" band was developed in UV-light, and the spot of brown color in the area of R_f = 0.35-0.55 was observed. The sorbent was carefully removed from the plate part with the area of 3 × 1 cm at the level of the "testifier" into a glass bottle with 10.00 mL of 0.01 M hydrochloric acid solution, the bottle content was vortexed for 5 min and filtered through the paper filter wetted with 0.01 M hydrochloric acid solution (eluate). **Method validation** – Schemes 4 and 5

The complete validation of the method developed was carried out using matrix (calibration and model) samples [1-9].

Stability

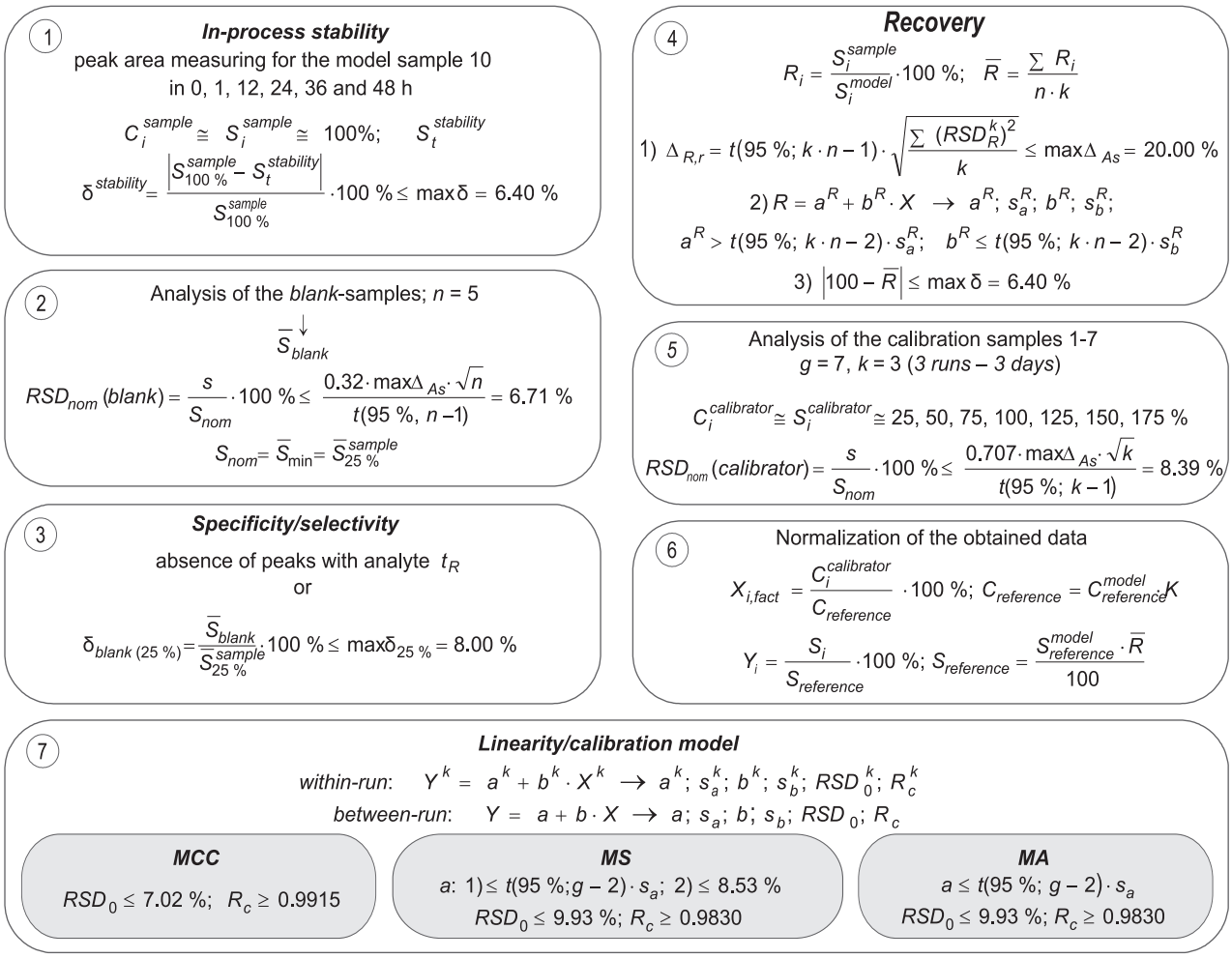
In process stability of metronidazole was verified in the way of chromatographing the eluate obtained for the model sample 10 – immediately and in 1, 12, 24, 36 and 48 hours after its preparation, and the systematic error $\delta^{stability}$ was calculated [9] and assessed.

Specificity/selectivity

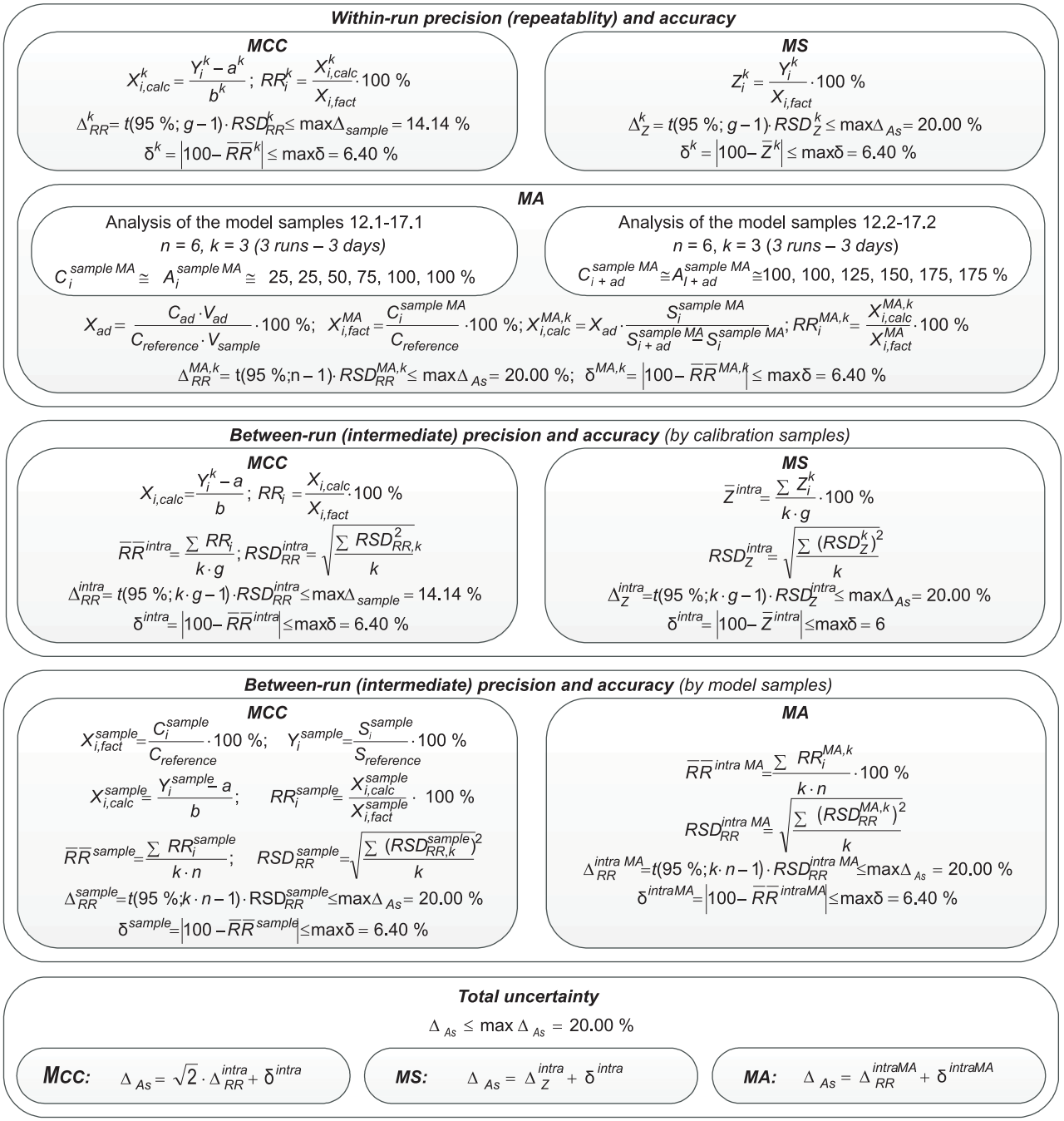
Blank-samples prepared using 5 different sources of blood or urine were analyzed; the summarized peaks areas within the range of t_R ± 0.5 min from the corresponding chromatograms were compared with the mean peak area of metronidazole from the chromatograms of the model samples 8 and 9. The value of δ_{blank} was calculated [9] to confirm the specificity/selectivity of the procedures of the analysis developed.

Recovery

Recovery *R* was determined at the levels of low, medium and high concentrations in the way of ana-



Scheme 4. The scheme of *stability*, *specificity*, *recovery* and *linearity* verification of HPLC/UV-procedures of metronidazole determination in blood and urine using calibration and model matrix samples



Scheme 5. The scheme of *precision*, *accuracy* verification and *total uncertainty* assessment of HPLC/UV-procedures of metronidazole determination in blood and urine using calibration and model matrix samples

lyzing the model samples 8-11 and comparing their peaks areas with the peaks areas for the model solutions 1-4, respectively. Reproducibility and significance of the recovery values were assessed [9].

The experiment described was carried out within at least 3 runs/days following the requirements to repeatability of peaks areas for replicate experiments [9].

Linearity/calibration model

To assess *linearity/calibration model* the calibration samples 1-7 were analyzed within 3 runs/days following the requirements to repeatability of peaks areas for replicate experiments [9]. The correlation coefficient, rest standard deviation and absolute term for linear dependences were calculated [3, 4, 9] as within-run (R^k_c, RSD^k_0, a^k) and between-run (R_c, RSD_0, a) parameters, and then compared with the corresponding acceptability criteria.

Accuracy and precision

MCC. The concentrations of calibration samples 1-7 and model samples 8-11 were recalculated using the corresponding within-run linear dependences or between-run linear dependence, and the values "found/given" RR_i were used to determine the confidence intervals Δ^k_{RR} (within-run precision), the total confidence intervals Δ^{intra}_{RR}, Δ^{sample}_{RR} (between-run precision),

the systematic errors δ^k (within-run accuracy), and the total systematic errors δ^{intra}, δ^{sample} (between-run accuracy) [5, 6, 9].

MS. The ratios Z_i for the calibration samples 1-7 were calculated and used to determine the confidence intervals $\Delta^k{}_Z$ (within-run precision), the total confidence interval Δ_Z^{intra} (between-run precision), the systematic errors δ^k (within-run accuracy) and the total systematic error δ^{intra} (between-run accuracy) [7, 9].

MA. The model samples 12.1-17.1 and 12.2-17.2 were analyzed within 3 runs/days. The concentrations of the model samples 12.1-17.1 were recalculated, and the values "found/given" RR_i^{MA} were used to determine the confidence intervals $\Delta_{RR}^{MA,\,k}$ (within-run precision), the total confidence interval $\Delta_{RR}^{intraMA}$ (between-run precision), the systematic errors $\delta^{MA,k}$ (within-run accuracy), and the total systematic error $\delta^{intra\ MA}$ (between-run accuracy) [8, 9].

The values of confidence intervals and systematic errors were compared with the corresponding acceptability criteria.

Limit of quantification (LOQ)

The lowest point on the calibration curve was accepted as LOQ [9].

Results and discussion

The HPLC/UV-method for metronidazole determination was proposed by authors before [22] and its specificity in relation to other 5-nitroimidazoles was shown. The suitability of the abovementioned analytical procedure for further work with biological fluids was assessed using the validation procedure by model solutions [22].

It was suggested to carry out metronidazole isolation from blood and urine by amphiphylic solvent extraction with the subsequent separation of the organic layer under the conditions of the aqueous phase saturation with an electrolyte; ammonium sulfate was used as an electrolyte.

Previously, blood and urine were processed with the corresponding acids (10 % trichloroacetic acid solution for blood and 0.1 M hydrochloric acid solution for urine). This way for processing biological fluids is accepted in the Ukrainian forensic and toxicological laboratories for the general analysis. Our modification of these sample preparation procedures is dilution of blood with water in 3 times before processing with 10 % trichloroacetic acid solution – to reduce the analyte co-precipitation due to decrease of the contact area between the analyte and blood cells.

To choose the optimal isolation conditions such amphiphylic solvents as 2-propanol and acetonitrile were used in the experiment. Owing to metronidazole amphoteric properties and proceeding from our results [23] isolation was carried out in the strong acid (pH = 2), neutral (pH = 7) and weak alkaline (pH = 9) medium; carrying out isolation of analytes from biological objects in the weak acid, neutral or weak alkaline medium (instead of the strong acid or alkaline medium) resulted in decreasing of co-extraction processes of the biological matrix components in a number of cases [24]. It is necessary to note that the shift of the pH real value in alkaline side was observed for the mixtures of electrolytes saturated solutions with amphiphylic solvents [25].

Thus, 6 sample preparation procedures were studied.

To find the optimal conditions of sample preparation we determined such validation parameters as specificity/selectivity and recovery according to Scheme 4.

Method validation

The validation provides application of the normalized coordinates [26]:

$$X_i = \frac{C_i}{C_{st}} \cdot 100\ \%;\quad Y_i = \frac{A_i}{A_{st}} \cdot 100\ \%, \qquad (1)$$

i. e. transition from the equation $A_i\ (S_i) = b_1 \cdot C_i + a_1$ 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 specific character of the method of analysis.

The analytical range D of the methods application is 25-175 % [9]; as 100 % the mean therapeutic metronidazole concentration in blood [19] is taken; the number of concentration levels g equals 7 in constant increments of 25 % [9].

Acceptability criteria for validation parameters were formed on the basis of systematic application of the "insignificance concept" [26] – the confidence interval Δ_2 was insignificant compared with the confidence interval Δ_1 at the conventional level p = 95 %, if the following inequality was correct:

$$\Delta_2 \le 0.32 \cdot \Delta_1, \qquad (2)$$

and proceeding from the value of extreme uncertainty Δ_{As} for the method in analytical toxicology, which equaled 25 % and 20 % [19, 20] – for the lowest point of the analytical range of the method application and for the rest of range.

Thus, the acceptability criterion for accuracy was as follows:

$$\max\delta = 0.32 \cdot \max\Delta_{As} = 0.32 \cdot 20.00\ \% = 6.40\ \%. \qquad (3)$$

In the MCC the acceptability criteria for the linear dependence and precision were found proceeding from the equality of uncertainty of plotting the calibration curve Δ_{cal} and uncertainty of the analysis of the sample to be analyzed Δ_{sample} [26], whence it was as follows:

$$\max\Delta_{cal} = \max\Delta_{sample} = \frac{\max\Delta_{As}}{\sqrt{2}} = \\ = 0.707 \cdot \max\Delta_{As} = 0.707 \cdot 20.00\ \% = 14.14\ \%. \qquad (4)$$

The method of validation by matrix samples consists of two phases [9]:

- the preliminary phase – determination and estimation of *in process stability* of the analyte in the solution to be analyzed, *specificity/selectivity* and *recovery* for the procedure;
- the main phase – determination and estimation of *linearity, accuracy, precision* and determination of *LOQ* for the procedure.

This method also contains the total uncertainty assessment.

For normalization of the experimental data obtained the same reference solution with the analyte concentration of $C^{model}_{reference} = C^{model}_{100\,\%}$ was used, but its peak area was corrected taking into account the value of recovery *R* (its significance and value were shown at the preliminary stage of validation).

Stability

The results of verification of *in process stability* of metronidazole in the solution to be analyzed showed the necessity to carry out all measurements within 12 hours after obtaining the solutions to be analyzed; in 12 hours the systematic error was high enough, but within the acceptability criteria; in 24 hours the systematic error increased significantly.

These data were taken into account when determining all validation parameters and should be used in the sequel.

Specificity/selectivity and recovery

The results of analysis of blank-samples and the assessment of systematic error caused by matrix influence were acceptable for all variants of the sample preparation procedures – we fixed the absence of peaks with the retention time, which was coincident with (or near to) the metronidazole retention time, on the chromatograms of blank-samples.

Subjectively, the procedures 1-2 and 2-2 (proceeding with amphiphylic solvents at pH = 7) were characterized by the lowest level of co-extractive substances by the picture of TLC-purification, but the procedures 1-1 and 2-1 (proceeding with amphiphylic solvents in the strong acid medium) had the worst results.

Recovery values determined in the preliminary phase of validation were reproducible for all procedures of analysis studied. But efficacy of metronidazole isolation was variable – from 85 % (procedure 2-1) to 97 % (proceeding with acetonitrile at pH = 2 and 9 for urine).

The total results of *recovery* determination for all procedures studied are given in Tab. 1.

Thus, based on the complex results of the *specificity/selectivity* and *recovery* assessment the procedures of the sample preparation, which include acetonitrile or 2-propanol extraction in the weak alkaline (pH = 9) medium followed by separation of the organic layer under the conditions of the aqueous phase saturation with ammonium sulfate are optimal and recommended for application by us.

Linearity/calibration model, accuracy and precision

The results of determination of R_c, RSD_0, a were positive for all variants of the sample preparation procedures and the solvent used, as well as for MCC, MS and MA (Tab. 2).

The values of *accuracy* and *precision* in the variant of MCC, MS and MA for all procedures studied are presented in Tab. 3-5.

The results of determination of accuracy and precision are the evidence of acceptable systematic and random errors of the HPLC/UV-procedures studied. Concerning MCC, MS and MA application – we observe the following tendency: MS – the best precision and the worst accuracy, MA – the best accuracy and the worst precision, but the difference is less distinct and clear; MCC is the optimal variant.

Limit of quantification (LOQ)

LOQ of the procedures developed is 5 μg/mL of metronidazole in biological sample.

Table 1

The results of recovery assessment for metronidazole determination procedures in blood and urine by the method of HPLC/UV

Parameter	procedure 1-1		procedure 1-2		procedure 1-3		procedure 2-1		procedure 2-2		procedure 2-3		Acceptability criteria
	blood	urine	blood	urine	blood	urine	blood	urine	blood	urine	blood	urine	
$\overline{R}$	91.34	94.97	92.28	94.20	90.26	94.68	85.89	95.48	90.12	97.68	92.88	97.22	–
$\Delta_{R,r}$	5.03	5.03	5.02	5.03	5.03	5.03	5.03	5.03	5.03	5.03	5.03	5.03	≤ 20.00 %
$\lvert 100 - \overline{R} \rvert$	8.66	5.03	7.72	5.80	9.74	5.32	14.11	4.52	9.88	2.32	7.12	2.78	≤ 6.40 %
b^R	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	$b^R \le t(95\,\%; k \cdot n - 2) \cdot s^R_b$
s^R_b	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	
a^R	90.65	94.26	91.60	93.50	89.58	93.98	85.25	94.77	89.45	96.95	92.18	96.49	$a^R > t(95\,\%; k \cdot n - 2) \cdot s^R_a$
s^R_a	1.27	1.32	1.28	1.31	1.26	1.32	1.20	1.33	1.26	1.36	1.29	1.35	

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Table 2

The results of linearity verification for metronidazole determination procedures in blood and urine by the method of HPLC/UV

Parameter	2-propanol								acetonitrile								Acceptability criteria		
	blood				urine				blood				urine						
	run 1	run 2	run 3	mean	run 1	run 2	run 3	mean	run 1	run 2	run 3	mean	run 1	run 2	run 3	mean	MCC	MS	MA
extraction at pH = 2																			
b	1.003	1.003	1.025	1.010	1.065	1.043	1.042	1.050	1.006	1.006	1.028	1.013	1.048	1.049	1.071	1.056	–	–	–
s_b	0.016	0.020	0.009	0.011	0.009	0.021	0.016	0.012	0.016	0.020	0.009	0.011	0.017	0.021	0.009	0.012	–	–	–
a	–2.455	–2.110	–4.232	–2.932	–4.401	–2.191	–2.553	–3.048	–2.462	–2.117	–4.247	–2.942	–2.565	–2.205	–4.423	–3.065	–	≤ 8.53 %	
s_a	1.767	2.274	0.962	1.280	1.000	2.364	1.837	1.331	1.772	2.281	0.965	1.284	1.847	2.377	1.005	1.338	–	$a \le 2.015 \cdot s_a$	
RSD_0	2.091	2.691	1.138	1.514	1.184	2.797	2.174	1.574	2.097	2.699	1.141	1.519	2.185	2.813	1.189	1.583	≤ 7.02 %	≤ 9.93 %	
R_c	0.9994	0.9990	0.9998	0.9997	0.9998	0.9990	0.9994	0.9997	0.9994	0.9990	0.9998	0.9997	0.9994	0.9990	0.9998	0.9997	≥ 0.9915	≥ 0.9830	
extraction at pH = 7																			
b	1.051	1.035	1.014	1.033	1.034	1.035	1.057	1.042	1.078	1.055	1.056	1.063	1.031	1.010	1.009	1.017	–	–	–
s_b	0.038	0.009	0.021	0.019	0.016	0.021	0.009	0.012	0.009	0.017	0.021	0.012	0.009	0.020	0.016	0.012	–	–	–
a	–4.624	–4.274	–2.132	–3.677	–2.531	–2.176	–4.364	–3.024	–4.454	–2.584	–2.221	–3.087	–4.258	–2.124	–2.471	–2.951	–	≤ 8.53%	
s_a	4.301	0.973	2.297	2.071	1.822	2.345	0.992	1.320	1.012	1.860	2.393	1.347	0.968	2.290	1.779	1.288	–	$a \le 2.015 \cdot s_a$	
RSD_0	5.089	1.151	2.718	2.450	2.156	2.775	1.174	1.562	1.197	2.200	2.832	1.594	1.145	2.709	2.104	1.524	≤ 7.02 %	≤ 9.93%	
R_c	0.9967	0.9998	0.9990	0.9992	0.9994	0.9990	0.9998	0.9997	0.9998	0.9994	0.9990	0.9997	0.9998	0.9990	0.9994	0.9997	≥ 0.9915	≥ 0.9830	
extraction at pH = 9																			
b	1.038	1.089	1.057	1.062	1.039	1.040	1.062	1.047	1.028	1.060	1.018	1.035	1.004	1.005	1.026	1.012	–	–	–
s_b	0.024	0.024	0.017	0.003	0.016	0.021	0.009	0.012	0.019	0.020	0.022	0.014	0.016	0.020	0.009	0.011	–	–	–
a	1.587	–4.460	–1.635	–1.503	–2.543	–2.187	–4.387	–3.039	–2.497	–5.196	–1.790	–3.161	–2.459	–2.115	–4.239	–2.938	–	≤ 8.53%	
s_a	2.739	2.711	1.937	0.307	1.831	2.358	0.996	1.326	2.163	2.244	2.451	1.578	1.770	2.278	0.963	1.282	–	$a \le 2.015 \cdot s_a$	
RSD_0	3.241	3.208	2.292	0.364	2.167	2.790	1.179	1.569	2.560	2.655	2.900	1.868	2.095	2.696	1.139	1.517	≤ 7.02 %	≤ 9.93%	
R_c	0.9986	0.9988	0.9993	1.0000	0.9994	0.9990	0.9998	0.9997	0.9991	0.9991	0.9988	0.9995	0.9994	0.9990	0.9998	0.9997	≥ 0.9915	≥ 0.9830	

Table 3

The results of accuracy and precision verification for metronidazole determination procedures in blood and urine by the method of HPLC/UV (extraction at pH = 2)

Parameter	2-propanol						acetonitrile						Acceptability criteria
	blood			urine			blood			urine			
	run 1	run 2	run 3	run 1	run 2	run 3	run 1	run 2	run 3	run 1	run 2	run 3	
within-run accuracy and precision (MCC)													
$\overline{RR}^k$	100.77	100.50	100.31	100.26	100.91	100.88	100.33	100.31	100.58	100.73	100.46	100.27	–
δ^k	0.77	0.50	0.31	0.26	0.91	0.88	0.33	0.31	0.58	0.73	0.46	0.27	≤ 6.40 %
RSD^k_{RR}	3.43	3.72	2.08	1.80	5.08	4.30	2.73	3.92	3.58	3.23	3.83	1.85	–
Δ^k_{RR}	6.67	7.23	4.04	3.50	9.87	8.36	5.30	7.62	6.96	6.28	7.44	3.59	≤ 14.14 %
between-run accuracy and precision by calibration samples (MCC)													
$\overline{RR}^{intra}$	100.52			100.68			100.41			100.49			–
δ^{intra}	0.52			0.68			0.41			0.49			≤ 6.40 %
RSD^{intra}_{RR}	3.46			4.09			3.42			3.39			–
Δ^{intra}_{RR}	5.97			7.06			5.90			5.85			≤ 14.14 %
between-run accuracy and precision by model samples (MCC)													
$\overline{RR}^{sample}$	101.29			101.65			99.95			100.99			–
δ^{sample}	1.29			1.65			0.05			0.99			≤ 6.40 %
RSD^{sample}_{RR}	3.37			3.82			2.79			3.16			–
Δ^{sample}_{RR}	6.05			6.86			5.01			5.67			≤ 20.00 %
within-run accuracy and precision (MS)													
$\overline{Z}^k$	97.39	97.70	96.50	100.58	100.69	101.86	98.63	98.76	97.82	101.97	102.31	101.06	–
δ^k	2.61	2.30	3.50	0.58	0.69	1.86	1.37	1.24	2.18	1.97	2.31	1.06	≤ 6.40 %
RSD^k_Z	1.79	3.31	3.99	3.99	4.05	2.75	2.50	3.93	2.76	1.81	3.69	4.05	–
Δ^k_Z	3.48	6.43	7.75	7.75	7.87	5.34	4.86	7.64	5.36	3.52	7.17	7.87	≤ 20.00 %
between-run accuracy and precision (MS)													
$\overline{Z}^{intra}$	97.20			101.04			98.40			101.78			–
δ^{intra}	2.80			1.04			1.60			1.78			≤ 6.40 %
RSD^{intra}_Z	3.17			3.65			3.13			3.33			–
Δ^{intra}_Z	5.47			6.30			5.40			5.74			≤ 20.00 %
within-run accuracy and precision (MA)													
$\overline{RR}^{MA,k}$	99.52	98.99	100.32	103.09	100.10	97.79	103.41	100.11	99.39	98.50	100.89	102.08	–
$\delta^{MA,k}$	0.48	1.01	0.32	3.09	0.10	2.21	3.41	0.11	0.61	1.50	0.89	2.08	≤ 6.40 %
$RSD^{MA,k}_{RR}$	3.49	4.06	3.70	9.40	4.70	5.39	10.15	3.14	5.04	3.47	3.34	8.20	–
$\Delta^{MA,k}_{RR}$	7.03	8.18	7.46	18.93	9.47	10.87	20.44	6.32	10.16	6.99	6.72	16.53	≤ 20.00 %
between-run accuracy and precision (MA)													
$\overline{RR}^{intraMA}$	99.61			100.33			100.97			100.49			–
$\delta^{intraMA}$	0.39			0.33			0.97			0.49			≤ 6.40 %
$RSD^{intra\,MA}_{RR}$	3.76			6.82			6.79			5.49			–
$\Delta^{intraMA}_{RR}$	6.54			11.86			11.81			9.55			≤ 20.00 %

Table 4

The results of accuracy and precision verification for metronidazole determination procedures in blood and urine by the method of HPLC/UV (extraction at pH = 7)

Parameter	2-propanol						acetonitrile						Acceptability criteria
	blood			urine			blood			urine			
	run 1	run 2	run 3	run 1	run 2	run 3	run 1	run 2	run 3	run 1	run 2	run 3	
within-run accuracy and precision (MCC)													
$\overline{RR}^k$	101.04	100.31	100.50	100.73	99.40	100.72	99.73	99.86	100.70	100.70	100.58	100.70	–
δ^k	1.04	0.31	0.50	0.73	0.60	0.72	0.27	0.14	0.70	0.70	0.58	0.70	≤ 6.40 %
RSD^k_{RR}	5.80	2.09	3.72	3.21	4.43	3.67	5.70	4.24	4.50	3.07	4.61	3.39	–
Δ^k_{RR}	11.27	4.06	7.23	6.24	8.61	7.13	11.08	8.24	8.74	5.97	8.96	6.59	≤ 14.14 %
between-run accuracy and precision by calibration samples (MCC)													
$\overline{RR}^{intra}$	100.62			100.29			100.10			100.66			–
δ^{intra}	0.62			0.29			0.10			0.66			≤ 6.40 %
RSD^{intra}_{RR}	4.18			3.12			4.45			4.11			–
Δ^{intra}_{RR}	7.20			5.38			7.67			7.09			≤ 14.14 %
between-run accuracy and precision by model samples (MCC)													
$\overline{RR}^{sample}$	101.36			100.14			98.46			101.07			–
δ^{sample}	1.36			0.14			1.54			1.07			≤ 6.40 %
RSD^{sample}_{RR}	4.07			2.74			2.64			3.86			–
Δ^{sample}_{RR}	7.31			4.92			4.75			6.93			≤ 20.00 %
within-run accuracy and precision (MS)													
$\overline{Z}^k$	99.34	97.51	98.71	100.62	103.14	98.64	99.90	99.72	98.45	102.15	102.90	101.57	–
δ^k	0.66	2.49	1.29	0.62	3.14	1.36	0.10	0.28	1.55	2.15	2.90	1.57	≤ 6.40 %
RSD^k_Z	4.24	4.03	3.34	1.79	3.73	4.18	5.47	4.60	3.69	1.87	4.46	4.73	–
Δ^k_Z	8.24	7.83	6.49	3.48	7.25	8.12	10.63	8.94	7.17	3.63	8.67	9.19	≤ 20.00 %
between-run accuracy and precision (MS)													
$\overline{Z}^{intra}$	98.52			100.80			99.35			102.21			–
δ^{intra}	1.48			0.80			0.65			2.21			≤ 6.40 %
RSD^{intra}_Z	3.89			3.39			4.65			3.91			–
Δ^{intra}_Z	6.71			5.85			8.02			6.74			≤ 20.00 %
within-run accuracy and precision (MA)													
$\overline{RR}^{MA,k}$	102.45	98.62	100.06	102.32	99.53	103.72	102.53	98.73	97.97	97.29	99.54	100.42	–
$\delta^{MA,k}$	2.45	1.38	0.06	2.32	0.47	3.72	2.53	1.27	2.03	2.71	0.46	0.42	≤ 6.40 %
$RSD^{MA,k}_{RR}$	6.41	3.17	3.38	8.76	4.00	11.05	10.20	4.96	6.48	4.70	3.18	5.06	–
$\Delta^{MA,k}_{RR}$	12.92	6.39	6.82	17.65	8.07	22.26	20.55	9.99	13.06	9.47	6.42	10.20	≤ 20.00 %
between-run accuracy and precision (MA)													
$\overline{RR}^{intraMA}$	100.38			101.86			99.74			99.08			–
$\delta^{intraMA}$	0.38			1.86			0.26			0.92			≤ 6.40 %
$RSD^{intra\,MA}_{RR}$	4.57			8.46			7.54			4.39			–
$\Delta^{intraMA}_{RR}$	7.95			14.72			13.12			7.64			≤ 20.00 %

Table 5

The results of accuracy and precision verification for metronidazole determination procedures in blood and urine by the method of HPLC/UV (extraction at pH = 9)

Parameter	2-propanol						acetonitrile						Acceptability criteria
	blood			urine			blood			urine			
	run 1	run 2	run 3	run 1	run 2	run 3	run 1	run 2	run 3	run 1	run 2	run 3	
within-run accuracy and precision (MCC)													
$\overline{RR}^k$	98.91	99.58	100.46	100.71	100.09	100.43	100.59	100.43	100.45	100.60	100.65	99.87	–
δ^k	1.09	0.42	0.46	0.71	0.09	0.43	0.59	0.43	0.45	0.60	0.65	0.13	≤ 6.40 %
RSD^k_{RR}	5.88	2.92	3.18	3.11	3.99	2.98	3.39	3.01	3.89	3.37	4.14	1.74	–
Δ^k_{RR}	11.43	5.67	6.18	6.04	7.75	5.79	6.59	5.85	7.56	6.55	8.04	3.38	≤ 14.14 %
between-run accuracy and precision by calibration samples (MCC)													
$\overline{RR}^{intra}$	99.66			100.41			100.49			100.37			–
δ^{intra}	0.34			0.41			0.49			0.37			≤ 6.40 %
RSD^{intra}_{RR}	3.57			3.70			3.80			3.41			–
Δ^{intra}_{RR}	6.15			6.39			6.56			5.88			≤ 14.14 %
between-run accuracy and precision by model samples (MCC)													
$\overline{RR}^{sample}$	97.34			100.55			100.83			100.59			–
δ^{sample}	2.66			0.55			0.83			0.59			≤ 6.40 %
RSD^{sample}_{RR}	2.86			3.23			3.51			2.83			–
Δ^{sample}_{RR}	5.14			5.80			6.31			5.09			≤ 20.00 %
within-run accuracy and precision (MS)													
$\overline{Z}^k$	106.47	103.75	104.24	101.25	102.17	100.64	99.74	98.73	99.58	98.23	98.43	96.20	–
δ^k	6.47	3.75	4.24	1.25	2.17	0.64	0.26	1.27	0.42	1.77	1.57	3.80	≤ 6.40 %
RSD^k_Z	4.28	5.34	2.83	1.83	4.29	4.92	2.47	4.84	3.68	2.37	3.34	3.69	–
Δ^k_Z	8.32	10.38	5.50	3.56	8.34	9.56	4.80	9.40	7.15	4.61	6.49	7.17	≤ 20.00 %
between-run accuracy and precision (MS)													
$\overline{Z}^{intra}$	104.82			101.35			99.35			97.62			–
δ^{intra}	4.82			1.35			0.65			2.38			≤ 6.40 %
RSD^{intra}_Z	4.28			3.91			3.79			3.18			–
Δ^{intra}_Z	7.38			6.74			6.54			5.48			≤ 20.00 %
within-run accuracy and precision (MA)													
$\overline{RR}^{MA,k}$	98.81	98.74	96.61	99.02	97.79	97.99	98.30	102.18	103.16	101.36	99.46	102.22	–
$\delta^{MA,k}$	1.19	1.26	3.39	0.98	2.21	2.01	1.70	2.18	3.16	1.36	0.54	2.22	≤ 6.40 %
$RSD^{MA,k}_{RR}$	3.16	4.77	3.80	4.18	5.40	7.70	5.51	8.41	9.55	6.64	5.22	7.56	–
$\Delta^{MA,k}_{RR}$	6.37	9.62	7.66	8.43	10.88	15.51	11.10	16.94	19.25	13.39	10.51	15.23	≤ 20.00 %
between-run accuracy and precision (MA)													
$\overline{RR}^{intraMA}$	98.05			98.27			101.21			101.01			–
$\delta^{intraMA}$	1.95			1.73			1.21			1.01			≤ 6.40 %
$RSD^{intra\,MA}_{RR}$	3.97			5.94			8.01			6.54			–
$\Delta^{intraMA}_{RR}$	6.90			10.34			13.93			11.38			≤ 20.00 %

Total uncertainty

The results of the total uncertainty assessment show the acceptability of the procedures developed. The least values of the total uncertainty are fixed for MCC; for MS and MA they are at the same level.

Conclusions

1. The HPLC/UV-procedures of metronidazole quantitative determination in blood and urine using the standard sample preparation with application of amphiphylic solvent (acetonitrile and 2-propanol) for the analyte isolation at pH = 2, 7 and 9 with further separation of the organic layer under the conditions of the aqueous phase saturation by ammonium sulfate have been developed.

2. Validation of the procedures developed has been carried out using calibration and model samples by such parameters as stability, specificity calibration model, accuracy and precision using different analytical and standardization methods – MCC, MS and MA; and application of the validation scheme possibility offered by us before has been confirmed.

3. The HPLC/UV-procedures of metronidazole quantitative determination developed satisfy the acceptability criteria for all validation parameters. Carrying out the preliminary phase of validation allowed us to eliminate the insufficient sample preparation and avoid fulfillment of the main validation phase for these procedures.

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

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