ANALYTICAL METHODS IN CHEMISTRY AND CHEMICAL TECHNOLOGY

АНАЛИТИЧЕСКИЕ МЕТОДЫ В ХИМИИ И ХИМИЧЕСКОЙ ТЕХНОЛОГИИ

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RESEARCH ARTICLE

Validation of a method for the quantitative determination of narcotic and psychotropic substances in urine by UHPLC–MS/MS

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Abstract

Objectives. *To validate a new method for the quantitative determination of 31 potent and narcotic substances and their metabolites in urine that meets the requirements of ISO/IEC 17025 using a fast and highly sensitive method of chromato-mass spectrometry with a view to introducing such a method into the routine practice of the National Anti-Doping Laboratory of the Lomonosov Moscow State University (NADL MSU).*

Methods. *Urine samples soldered with standard solutions were analyzed using ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS).*

Results. *Diagnostic precursor/ion-product pairs and collision energies were established to allow unambiguous identification of the analyzed substances. During sample preparation, hydrolysis conditions were optimized. Selectivity, linearity, limits of qualitative determination, limit of quantitative determination (established under the contract with the customer firm), matrix effect, and measurement uncertainty were defined. Systematized data grouped by classes of analytes are given in the final table.*

Conclusions. *The important advantages of the presented technique are the absence of complex and lengthy sample preparation, as well as the short time of the analysis method (about 10 min), which can significantly reduce duration along with labor and analysis costs. The addition of new analytes will ensure the versatility of the technique, as well as expanding its scope.*

Keywords: *UHPLC–MS/MS, GC–MS/MS, validation, quantitation, narcotic potent and psychotropic substances*

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НАУЧНАЯ СТАТЬЯ

Валидация методики количественного определения наркотических и психотропных веществ в моче методом СВЭЖХ-МС/МС

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Аннотация

Цели. *Валидировать и ввести в рутинную практику НАДЛ МГУ новую, отвечающую требованиям ISO/IEC 17025, методику количественного определения 31 сильнодействующих и наркотических вещества и их метаболитов в моче с использованием быстрого и высокочувствительного метода хромато-масс-спектрометрии.*
Методы. *Анализ спайкованных с растворами стандартов образцов мочи проводили методом сверхэффективной жидкостной хроматографии–тандемной масс-спектрометрии (СВЭЖХ-МС/МС).*
Результаты. *В работе установлены диагностические пары прекурсор/ион-продукт и найдены энергии соударения, позволяющие однозначно идентифицировать анализируемые вещества; оптимизированы условия гидролиза при проведении пробоподготовки; определены селективность, линейность, предел качественного определения, предел количественного определения (установлен в рамках договора с фирмой-заказчиком), эффект матрицы и неопределенность измерения. Систематизированные данные приведены в итоговой таблице и сгруппированы по классам определяемых веществ.*

Выводы. *Представленная методика обладает важными преимуществами – отсутствием сложной и продолжительной пробоподготовки, а также коротким временем метода анализа – около 10 мин, что позволяет существенно снизить трудозатраты, продолжительность и себестоимость анализа. Дополнение новыми определяемыми веществами обеспечит ее универсальность и позволит расширить область применения.*

Ключевые слова: *СВЭЖХ-МС/МС, ГХ-МС/МС, валидация, количественное определение, наркотические сильнодействующие и психотропные вещества*

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INTRODUCTION

In recent decades, the emergence of new narcotic and psychotropic substances, which has been accompanied by a steady increase in their abuse, has become a global problem. According to information provided by the United Nations International Narcotics Control Board in 2020[1], despite the COVID-19 pandemic, which has created unprecedented challenges for the supply of controlled medicines and global health systems in general, the number of seizures of potent narcotic substances has remained at a stable high level. Meanwhile, the number of electronic marketplaces on the wider Internet—and in particular on the so-called "dark web"—has increased. Encrypted secure applications and social networks have begun to play a significant role in the search for such substances at the consumer level. In view of this, the relevance of chemical and toxicological analysis in order to control the intake of illegal substances has increased significantly. As a result of the active use of narcotic drugs and psychotropic substances, the number of people with drug addiction is steadily growing, while the number of severe intoxications leading to death is also increasing [1].

In this regard, one of the most urgent and significant tasks of modern toxicological analysis is the development of new, express, and accurate techniques, as well as improving already used methods for detecting controlled substances and their metabolites in objects of biological origin, as well as supporting their validation and implementation in the routine practice of laboratories.

To date, chromatographic methods of determination are widely used in combination with mass spectrometry in various chemical and toxicological studies, providing high rates of selectivity and sensitivity [2–4]. Although gas chromatography-mass spectrometry (GC–MS/MS) methods have long been used for routine analysis, the associated sample preparation, including the additional purification, extraction, and derivatization for low-volatile organic compounds, is time-consuming. For this reason, ultra high performance liquid chromatography in combination with tandem mass spectrometry (UHPLC–MS/MS) has become a popular method for screening analysis to determine the presence of narcotic, potent, and psychotropic substances [5].

Previously, the Anti-Doping Center—the predecessor of the National Anti-Doping Laboratory of M.V. Lomonosov Moscow State University (NADL MSU)—had introduced a method for toxicological monitoring of urine samples to identify classes of opiates, stimulants, cannabinoids, barbiturates, and benzodiazepines. The World Anti-Doping Agency (WADA) criteria for the analysis of urine samples

[1] Report of the International Narcotics Control Boards (INCB) for 2020. United Nations: International Narcotics Control Board). Vein; 2021. 167 p. URL: https://www.incb.org/documents/Publications/AnnualReports/AR2020/Annual_Report/E_INCB_2020_1_rus.pdf (Accessed February 10, 2022).

were taken as the basis for conducting method validation from the TD_IDCR and TD_DL technical documents associated with this period.[2,3] Although screening analysis and confirmation procedures for most compounds were performed by UHPLC–MS/MS, some substances and their metabolites were determined by GC–MS/MS.

In order to unify the analysis of substances using the UHPLC–MS/MS method and bring the methodology in line with the new regulatory documents[4], in 2020, the Laboratory revalidated the methodology for the quantitative determination of substances listed in Table 1. As a result, sample preparation was optimized, allowing the UHPLC–MS/MS parameters to be grouped according to the classes of analytes. Compliance of the method's validation with the requirements of the new version of ISO/IEC 17025 was confirmed by the Association of Analytical Centers "Analitika".[5]

The present study is aimed at developing an express and highly selective method for the quantitative determination of 31 substances and their metabolites included in the toxicological monitoring group.

The performance of chemico-toxicological analysis of employees of enterprises involved in work requiring increased attention, or in shift work around the clock, was carried out in accordance with the terms of the contract concluded with the customer company. The list of determined compounds is given in Table 1.

MATERIALS AND METHODS

Certified reference materials

For the experiments, certified standard samples with an initial concentration of 1.0 mg/mL were used: amphetamine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDEA), methylenedioxymethamphetamine (MDMA), methamphetamine, amobarbital, butabarbital, butalbital, pentabarbital, secobarbital, phenobarbital, alprazolam, clonazepam, lorazepam, midazolam, oxazepam, nordiazepam, temazepam, triazepam, flurazepam, benzoylecgonine, hydrocodone, hydromorphone, codeine, 6-monoacetylmorphine, morphine, oxycodone, oxymorphone, propoxyphene, methadone, and delta-9-tetrahydrocannabiol-9-acid (THC) purchased from *LGC* (United Kingdom).

Internal standards (ISTD) with an initial concentration of 0.01 mg/mL: demoxepam-d_5 (ISTD 1), morphine-d_3 (ISTD 2), and phenobarbital-d_5 (ISTD 3), purchased from *Cerilliant* (USA), bupranolol (ISTD 4) and mephruside (ISTD 5) purchased from *NMI* (Australia).

All manufacturers of certified reference materials meet the requirements of ISO 17034[6]. Manufacturers not accredited to ISO 17034 have documented the identity and purity of reference materials from competent laboratories that meet the requirements of ISO/IEC 17025 [9], which is confirmed by certificates of analysis.

Chemicals

Acetonitrile (*Merck*, Germany), methanol (*Merck*), and formic acid (*Acros Organics*, Belgium) were HPLC grade, deionized water for analysis (18.2 mΩ) was obtained using a Milli-Q system (*Millipore*, USA).

Sample preparation reagents: potassium carbonate (purity is 99% minimum), potassium bicarbonate (purity is 99% minimum), anhydrous sodium sulfate (purity is 99.5% minimum), and diethyl ether (purity is 95% minimum) manufactured by *Sigma-Aldrich* (USA); potassium dihydrogen phosphate (purity is not less than 99%), sodium phosphate dibasic dihydrate (purity is not less than 99%), and sodium azide (purity is not less than 99%) manufactured by *Merck*; *Escherichia Coli* K 12 β-glucuronidase (*Roche Diagnostics*, Switzerland); cartridges for solid phase extraction Oasis® MCX (*Waters*, USA).

Glass tubes with screw caps 16 × 125 mm (*Pyrex*, USA), 1.5 mL polypropylene tubes (*Eppendorf*, Germany), 2.0 mL glass vials (*Macherey-Nagel GmbH & Co*, Düren, Germany), 0.2 mL polypropylene vials (*Agilent Technologies*, USA), crimper, decapper, and gas tight caps for vials were used in the study.

Samples for analysis

Urine samples for chemico-toxicological analysis were taken from volunteers, each of whom provided a

[2] WADA Technical Document – TD2021IDCR. 2021. URL: https://www.wada-ama.org/en/resources/lab-documents/td2021idcr (Accessed February 11, 2022).

[3] WADA Technical Document – TD2021IDL. 2021. URL: https://www.wada-ama.org/en/resources/lab-documents/td2021dl (Accessed February 11, 2022).

[4] ISO/IEC 17025-2019. General requirements for the competence of testing and calibration laboratories. (In Russ.). URL: https://docs.cntd.ru/document/1200166732 (Accessed February 11, 2022).

[5] Association of Analytical Centers "Analytica." (In Russ.). URL: https://aac-analitica.ru/akkreditaciya.html (Accessed February 11, 2022).

[6] ISO 17034-2021 General requirements for the competence of reference material producers. (In Russ.). URL: https://docs.cntd.ru/document/1200181084 (Accessed February 11, 2022).

Table 1. Target compounds determined according to the program of chemico-toxicological analysis in accordance with the requirements of the customer company

Compounds/Metabolites	Thresholds, ng/mL
Amphetamine, Methylenedioxyamphetamine (MDA), Methylenedioxymethamphetamine (MDEA), Methylenedioxymethamphetamine (MDMA), Methamphetamine	250
Amobarbital, Butobarbital, Butalbital, Pentobarbital, Secobarbital, Phenobarbital	100
Alprazolam, Clonazepam, Lorazepam, Midazolam, Nordiazepam, Oxazepam, Temazepam, Triazolam, Flurazepam	100
Benzoylecgonine	100
Hydrocodone, Hydromorphone, Codeine, Morphine, Oxycodone, Oxymorphone	100
6-Monoacetylmorphine (6-MAM)	10
Methadone, Propoxyphene	200
Delta-9-tetrahydrocannabiol-9-acid (carboxy-THC)	10

written informed consent to the use of his biological material for scientific purposes, in accordance with the requirements of the laboratory code of ethics. The conducted studies do not contradict the Declaration of Helsinki of the World Medical Association.[7] These samples were previously examined for the absence of detectable components and used in the work as a certified negative urine control (blank urine, Blank).

Control samples were prepared for each group of compounds: a positive urine control containing the amounts of detectable compounds at the threshold level (positive control urine, PCU) and containing the amounts of detectable compounds with a given concentration different from the threshold value (LabQC). A certified negative urine control (Blank) was used as a matrix.

Equipment

In the study, the equipment was as follows: a triple quadrupole TSQ Vantage mass spectrometer (*Thermo Fisher Scientific*, San Jose, USA), in combination with a liquid UltiMate 3000 chromatograph (*Dionex*, Germany) (UHPLC–MS/MS), a thermostat-incubator with programmable temperature (*Binder*, Germany), low-temperature liquid thermostat (*Grant*, United Kingdom), solid-state incubator with programmable temperature (Grant), rotary stirrer, vortex V-1 plus (*BioSan*, Latvia), table centrifuge with Rotina horizontal rotor (*Hettich*, Germany), Discovery DV215CD (*OHaus*, USA) analytical balance (accuracy 5 digits), automatic batchers of variable volume (10–200 μL and 100–1000 μL) (*Eppendorf*, Germany), and 10-mL dispenser (*Brand*, Germany).

Sample preparation

Sample preparation included the following main steps: enzymatic hydrolysis, extraction, solvent removal, and resolubility for entry into the UHPLC–MS/MS system.

To perform sample preparation for the quantitative determination of target compounds, 12 tubes with screw caps (16 × 125 mm) were taken and labeled with Cal0 (Blank), Cal1, Cal2, Cal3, Cal4, Cal5, Cal6, and PCU markers in five repetitions. In the first 7 tubes, 1 mL of a certified negative urine control (Blank) was added, and in tubes 8–12, 1 mL of a urine sample containing the amount of analytes at the threshold level (PCU) was added.

The preparation of urine samples for validation was performed by two independent experts as follows: 20 μL of the mixture of ISTD 1–ISTD 3 internal standards were added to the tubes labeled

[7] Declaration of Helsinki. World Medical Association. URL: http://acto-russia.org/index.php?option=com_content&task=view&id=21 (Accessed February 11, 2022).

Cal0–Cal6 and PCU. Then, 1 mL of the hydrolysis buffer mixture (pH 7.4) was added to each tube and mixed on a Vortex contact mixer. Then the samples were placed in a thermostat-incubator with a programmed temperature of 57 ± 3°C for 60 ± 10 min. After that, the tubes were cooled to room temperature (25°C). Then 1 mL of carbonate buffer solution (pH 9–11) and 1–2 g of sodium sulfate were added to each tube and tubes were shaken for 5–10 s. Next, 5 mL of diethyl ether was added to each test tube, closed with a lid, and placed in a rotary mixer for 20 ± 5 min. Then, the tubes with the samples were centrifuged at 2700–3100 rpm for 3–4 min; were placed in a liquid thermostat with a programmable set temperature (−30°C) until the water layer was frozen (5–10 min). Next, the organic layer was transferred into test tubes 16 × 125 mm, evaporated to dryness in a solid-state heater at 70°C, 200 μL of a solution (Diluent) containing a 0.1% solution of formic acid in methanol/water = 5:95, *v*/*v*, was added to the dry residue with the internal standard solutions (ISTD 4, ISTD 5). Then, each tube was shaken on a Vortex mixer for 5–10 s, the obtained extracts were transferred into 0.2 mL polypropylene vials, closed with vial lids, and placed on the instrument.

Instrumental analysis

UHPLC–MS/MS analysis of the sample was performed under the following parameters: an Acquity BEH-C18 analytical column (100 mm, 2.1 mm, film thickness 1.7 μm, *Waters*, USA) was used. The flow rate of the mobile phase was 0.35 mL/min. The elution program started with a 0.5 min isocratic step at 95% of 0.1%-formic acid solution in water (A) and 5% of 0.1%-formic acid solution in methanol (B), followed by a linear increase to 95% solution B over 4.5 min, hold at 95% (B) for 2.5 min. The solution was then equilibrated to the end of the analysis for 10 min. The volume of the injected sample was 10 μL. Detection was performed in the mode of selective reaction monitoring (SRM) of positive and negative ions using a heated electrospray ion source (HESI II). The gas pressure in the impact chamber was 1.5 mTorr (argon 99.9995%). The evaporator temperature was 370°C, the capillary temperature was 350°C, and the spray voltage was 4000 V.

CHARACTERISTICS OF THE METHOD

The validation of the method was carried out in accordance with the requirements established in ISO/IEC 17025[8], GOST R 8.795-2012[9], the measurement uncertainty assessment guide ISO/IEC Guide 98-3:2008[10], as well as WADA technical documents TD2021IDCR and TD2021DL.

Selectivity

In order to investigate the potential interfering effect of the matrix, certified negative urine control (Blank) and control urine samples were prepared with the addition of a mixture of each group of compounds at the threshold level. There should be no interfering peaks of the matrix components with a signal-to-noise ratio exceeding 3:1 on the obtained mass chromatograms of the analytes in the corresponding intervals of scanning the retention time (RT) of the analytes within ± 0.1 min.

Linearity

In order to build a linear dependence of the quantitative determination of the components, a series of calibration solutions containing components in the range of 50–300% (Cal0–Cal6) of the threshold concentrations was conducted by sample preparation.

Based on the results of the analysis, we determined and built graphic dependences of the concentration on the received signal, the linearity of the calibration curves was evaluated using the correlation coefficients R^2, which should not be lower than 0.99.

Limits of quantitative and qualitative determinations

The limit of quantitation (LOQ) of a compound corresponds to the lowest concentration that falls within the linear range of the technique. In order

[8] ISO/IEC 17025-2019. General requirements for the competence of testing and calibration laboratories. (In Russ.). URL: https://docs.cntd.ru/document/1200166732 (Accessed February 11, 2022).

[9] GOST R 8.795-2012. National Standard of Russian Federation. State system for ensuring the uniformity of measurements. Identification of chemicals substance by a chromato-mass spectrometry method. The general requirements. (In Russ.). URL: https://docs.cntd.ru/document/1200102300 (Accessed February 11, 2022).

[10] ISO/IEC Guide 98-3:2008. Uncertainty of measurement. Part 3. Guide to the expression of uncertainty in measurement. 2008. URL: https://docs.cntd.ru/document/1200146871 (Accessed February 11, 2022).

to obtain this validation parameter, a certified negative urine control (Blank) was prepared with the addition of the least significant concentration of the calibration solution.

The limit of detection (LOD) of the method was set earlier during the development and validation of the primary analysis procedure by preparing a series of sequential dilutions of solutions in urine with a final concentration of analytes at a level of 10% (or less) of the threshold value.

Matrix effect

In order to assess the effect of urine components on the analyte determination (matrix effect, ME), control urine samples were studied with the addition of a mixture of each group of compounds at the threshold level and the corresponding solutions of the same analytes in a solvent with the same concentration. The influence of the urine matrix was evaluated by the formula (1):

$$\text{ME} = \frac{\text{Control peak area (PCU)}}{\text{Control solution peak area}} \times 100\% . \quad (1)$$

An ME value greater than 100% indicated an increase in ionization, and a value below 100% indicated suppression of ionization by the sample matrix.

Measurement uncertainty

The determination was carried out in accordance with the Guidelines for the Expression of Uncertainty of Measurement (GUM)[11], which establishes general rules for assessing and expressing measurement uncertainty in laboratories accredited to ISO/IEC 17025. When assessing uncertainty, an intralaboratory approach based on the determination of intermediate precision (intralaboratory reproducibility) was used. This approach consists of a three-component measurement model: the sum of measurements of the average value of the measurement method (m), estimates of the systematic error of the method (B), and the contribution of random error (e) (2):

$$y = m + B + e . \quad (2)$$

The combined standard uncertainty (u_c) was calculated as the root sum of the squares of the intermediate PCU precision (u_{prec}), the intermediate precision of the calibrators (u_{cal}), the bias uncertainty about the PCU setpoint in the presence of a systematic error (u_{bias}), and the uncertainty considering the analyte sample preparation process (u_{other}) according to the formula (3):

$$u_c = \sqrt{u_{prec}^2 + u_{cal}^2 + u_{bias}^2 + u_{other}^2} . \quad (3)$$

In order to assess the measurement uncertainty, a series of calibration solutions for each group of compounds and the corresponding positive urine controls containing the amounts of analytes at the threshold level were used.

RESULTS AND DISCUSSION

The main aim of this work was to develop and validate a rapid and reliable method for the quantitative determination of target compounds in urine samples. Precursor/product ion diagnostic pairs and collision energies were established to unambiguously identify the analyzed compounds. During the development of the method, optimal ionization conditions were obtained for each compound. The final data for the entire list of analytes are presented in Table 2.

Prior to validating the method, the conditions for the main stages of sample preparation, hydrolysis, and extraction were selected. Possibilities for using acid and enzymatic hydrolysis to determine compounds forming conjugates during metabolism were evaluated. The majority of the metabolites form derivatives of glucuronic acid, with only a small amount being excreted in the form of sulfates, acetates, and some other salts [6]. Although both types of hydrolysis are used to determine most of the substances during sample preparation, rather aggressive conditions are required when selecting an acid hydrolysis method: hydrochloric acid in high concentration (10N) and prolonged thermostating (at least 30 min) at high temperature (above 90°C), which may have a negative effect on the structures of some polar metabolites of the analyzed compounds (benzodiazepines, THC) [7]. By contrast, enzymatic hydrolysis does not require such conditions; instead,

[11] ISO/IEC Guide 98-3:2008. Uncertainty of measurement. Part 3. Guide to the expression of uncertainty in measurement. 2008. URL: https://docs.cntd.ru/document/1200146871 (Accessed February 11, 2022).

Table 2. Chromato-mass-spectrometric characteristics of analytes

Compound	RT, min	Type of ionization	Precursor ion, *m*/*z* (a.u.m.)	Product ion (collision energy), *m*/*z* (V)
Demoxepan-d_5 (ISTD 1)	3.60	+	292.1	180.1 (22)
				124.1 (37)
Morphine-d_3 (ISTD 2)	1.27	+	302.1	199.1 (25)
				128.1 (34)
Phenobarbital-d_5 (ISTD 3)	1.77	–	236.1	193.1 (13), 42.0 (20)
Bupranolol (ISTD 4)	4.10	+	272.1	216.1 (15)
				218.1 (15)
Mephruside (ISTD 5)	4.28	+	380.9	189.0 (30)
Amphetamine	2.78	+	136.1	119.1 (7)
				91.1 (16)
MDA	2.80	+	180.1	163.1 (10)
				135.1 (20)
MDMA	2.83	+	194.0	163.1 (12)
				135.1 (21)
MDEA	3.00	+	208.1	135.1 (21)
				163.1 (12)
Methamphetamine	2.85	+	150.1	91.1 (23)
				119.1 (10)
Amobarbital	3.66	–	225.1	42.1 (25)
				182.1 (10)
Butabarbital	4.35	–	201.1	168.1 (13)
				42.1 (20)
Butalbital	2.45	–	223.1	180.2 (10)
				42.1 (25)
Pentabarbital	3.52	–	225.1	42.1 (25)
				182.1 (10)
Secobarbital	4.60	–	237.1	42.1 (25)
				194.1 (10)
Phenobarbital	4.04	–	231.1	188.1 (12)
				42.1 (19)
Alprazolam	4.15	+	309.1	205.1 (41)
			311.1	205.1 (40)
Clonazepam	3.75	+	316.1	270.1 (24)
				214.1 (37)

Table 2. Continued

Compound	RT, min	Type of ionization	Precursor ion, *m*/*z* (a.u.m.)	Product ion (collision energy), *m*/*z* (V)
Lorazepam	4.10	+	323.1	277.1 (21)
			321.1	275.1 (21)
Midazolam	2.95	+	326.1	291.1 (26)
			328.1	291.1 (26)
Nordiazepam	4.37	+	271.1	140.1 (27)
				208.1 (27)
Oxazepam	4.10	+	287.1	241.1 (22)
			289.1	243.1 (21)
Temazepam	4.25	+	301.1	255.1 (29)
			303.1	257.1 (22)
Triazolam	4.14	+	343.1	308.1 (25)
			345.1	308.1 (25)
Flurazepam	2.90	+	388.1	315.1 (25)
			390.1	317.1 (22)
Benzoylecgonine	3.30	+	290.1	168.1 (19)
				105.1 (30)
Hydrocodone	2.73	+	300.1	128.1 (10)
				199.1 (11)
Hydromoron	1.87	+	286.1	157.1 (11)
				185.1 (20)
Codeine	2.58	+	300.1	152.1 (17)
				165.1 (14)
Morphine	1.36	+	386.1	152.1 (15)
				165.1 (8)
Oxycodone	2.67	+	316.1	241.1 (5)
				256.1 (20)
Oxymorphone	1.54	+	302.1	227.1 (11)
				284.1 (12)
6-MAM	2.76	+	328.1	165.1 (13)
				211.1 (12)
Propoxyphene	3.40	+	266.1	143.1 (19)
				128.1 (33)
Methadone	4.40	+	310.1	265.1 (14)
				219.1 (24)
Carboxy-THC	5.82	–	343.1	245.2 (31)
				191.2 (39)

E-coli beta-glucuronidase enzymes can be added to a phosphate buffer solution (pH 6.5–7.0) and incubation carried out at a temperature of 57°C for 60 min [8, 9]. Another important advantage of enzymatic hydrolysis is its high specificity due to the reduction in the urine matrix of interfering peaks associated with the cleavage of polysaccharide fragments of molecules, as well as the prevention of the breakdown of labile compounds and the exclusion of the use of aggressive media (reduction of hazard class). After taking the above factors into account, as well as the possible unification of sample preparation for all compounds, the choice was made in favor of enzymatic hydrolysis.

Some literature sources provide evidence that solid phase extraction (SPE) offers significant advantages as compared with liquid–liquid extraction (LLE) due to allowing purer eluates to be obtained [10]; this is especially important when determining substances at low concentrations. In the process of choosing the conditions for sample preparation, we compared the results after LLE of samples with diethyl ether and a carbonate buffer solution (pH 9.5–11) with added sodium sulfate with the results of SPE using Oasis® cartridges. Due to the threshold values for the quantitative determination of the validated compounds significantly exceeding the LOD (see Table 3), no significant difference was found in the analysis of the obtained extracts. Therefore, the choice was made in favor of LLE, which ultimately significantly reduced the cost and time of sample preparation for analysis.

Selectivity

An analysis of the mass chromatogram sections showed that the obtained mass chromatograms of the analytes in the corresponding intervals of scanning the RT of the analytes within ±0.1 min did not contain interfering peaks of the matrix components with a signal-to-noise ratio exceeding 3:1.

Linearity

For each analyte, a linear dependence of the concentration of a series of calibration solutions [11, 12], containing components in the range of 50–300% (Cal0–Cal6) of the threshold concentration value on the ratio of the signal of the analyte component to the signal of the corresponding internal standard was plotted. As an example, Figure shows a graph of a linear calibration curve for hydromorphone.

The results obtained indicate that the R^2 correlation coefficients for each compound were higher than the established value of 0.99 (the minimum value was 0.9918 for 6-MAM and the maximum value was 0.9992 for clonazepam and triazolam). The results obtained indicate a linear dependence in the selected concentration range (see Table 3).

Limit of quantitation

The obtained data on LOQ and LOD for each substance are presented in Table 3. LOQ and LOD values met the requirements of the customer's company (see Table 1).

Matrix effect

Most of the analyzed compounds showed ME values in the range of 85–115%, which indicates that the resulting matrix effect is negligible [13]. The minimum ME value was obtained for butalbital, 59.2 ± 7%, and the maximum for benzoylecgonine, 130.0 ± 2.0%. The data are given in Table 3.

Measurement uncertainty

The determination of intermediate precision was performed based on a data set of 5 PCU samples performed by two specialists within 5 days (n = 50). Each PCU result, in turn, was the average of three replicate measurements. The obtained data array was evaluated for outliers: the median of the sample, the lower and upper quartiles, and the inner and outer limits of the range were determined according to the method proposed in ISO/IEC Guide 98-3:2008. In a homogeneous sample of corrected (if necessary) values of the PCU samples, the mean, standard deviation, and relative standard deviation were determined under reproducibility conditions (intermediate precision, u_{prec}). The intermediate precision of the calibration solutions (u_{cal}) for each analyte was calculated as the root sum of the squares of the relative standard deviation of the levels of the calibration curve and the accuracy between the given and obtained concentration value for each level. The bias uncertainties about the PCU setpoint were evaluated in the presence of a systematic error (u_{bias}). The systematic error was determined using the Student's test. The uncertainty, which takes into account the process of sample preparation of u_{other} analytes, was calculated as the root sum of the squares of the uncertainties of the standard sample (according to the quality certificate),

Table 3. Validation characteristics of the methodology

Compound	LOD, ng/mL	LOQ*, ng/mL	Linearity, ng/mL	R^2 (n = 10)	ME, %	u_c, %
Amphetamine	0.5	125	125–750	0.9986 ± 0.0008	95.9 ± 5.1	6.5
MDA	2.0	125	125–750	0.9990 ± 0.0004	99.2 ± 4.6	6.2
MDEA	0.5	125	125–750	0.9983 ± 0.0012	101.5 ± 2.8	7.3
MDMA	0.5	125	125–750	0.9986 ± 0.0007	99.8 ± 3.3	7.6
Methamphetamine	0.5	125	125–750	0.9986 ± 0.0008	101.5 ± 3.9	6.7
Amobarbital	10	100	100–1000	0.9956 ± 0.0024	86.1 ± 5	8.8
Butabarbital	10	100	100–1000	0.9943 ± 0.0020	98.0 ± 5	8.7
Butalbital	10	100	100–1000	0.9950 ± 0.0020	59.2 ± 7	7.3
Pentabarbital	10	100	100–1000	0.9952 ± 0.0025	79.4 ± 6	8.6
Secobarbital	10	100	100–1000	0.9958 ± 0.0021	85.6 ± 8	8.3
Phenobarbital	10	50	50–2000	0.9979 ± 0.0014	86.1 ± 5	10.6
Alprazolam	0.5	50	50–300	0.9987 ± 007.00	94.7 ± 2.4	8.7
Clonazepam	0.5	50	50–300	0.9992 ± 0.0004	95.2 ± 4.6	7.1
Lorazepam	0.5	50	50–300	0.9987 ± 0.0006	106.0 ± 3.7	8.3
Midazolam	0.5	50	50–300	0.9987 ± 0.0005	92.1 ± 3.0	8.1
Oxazepam	0.5	50	50–300	0.9972 ± 0.0025	102.7 ± 4.3	9.8
Nordiazepam	0.5	50	50–300	0.9990 ± 0.0030	90.8 ± 3.7	7.2
Temazepam	0.5	50	50–300	0.9971 ± 0.0016	99.1 ± 4.3	8.2
Triazolam	0.5	50	50–300	0.9992 ± 0.0003	90.5 ± 2.1	6.8
Flurazepam	0.5	50	50–300	0.9980 ± 0.0013	100.8 ± 1.9	8.5
Benzoylecgonine	1.0	50	50–300	0.9989 ± 0.0006	130.0 ± 2.0	5.6
Hydrocodone	1.0	50	50–300	0.9971 ± 0.0030	105.1 ± 3.0	11.9
Hydromorphone	2.0	50	50–300	0.9989 ± 0.0003	109.6 ± 3.1	6.9

Table 3. Continued

Compound	LOD, ng/mL	LOQ*, ng/mL	Linearity, ng/mL	R^2 (n = 10)	ME, %	u_c, %
Codeine	1.0	50	50–300	0.9968 ± 0.0034	106.9 ± 2.6	12.7
Morphine	1.0	50	50–300	0.9987 ± 0.0006	100.3 ± 1.0	11. 6
Oxycodone	1.0	50	50–300	0.9959 ± 0.0038	100.9 ± 3.0	13.2
Oxymorphone	2.0	50	50–300	0.9965 ± 0.0024	95.6 ± 2.7	12.1
6-MAM	0.2	5	5–30	0.9918 ± 0.0025	97.5 ± 1.5	16.2
Propoxyphen	10	100	100–600	0.9985 ± 0.0010	112.2 ± 5.7	7.5
Methadone	0.5	100	100–600	0.9982 ± 0.0009	99.0 ± 4.5	6.9
Carboxy-THC	2.0	5	5–200	0.9983 ± 0.0012	95.2 ± 6	19.0

***** In accordance with the terms of the contract with the customer.

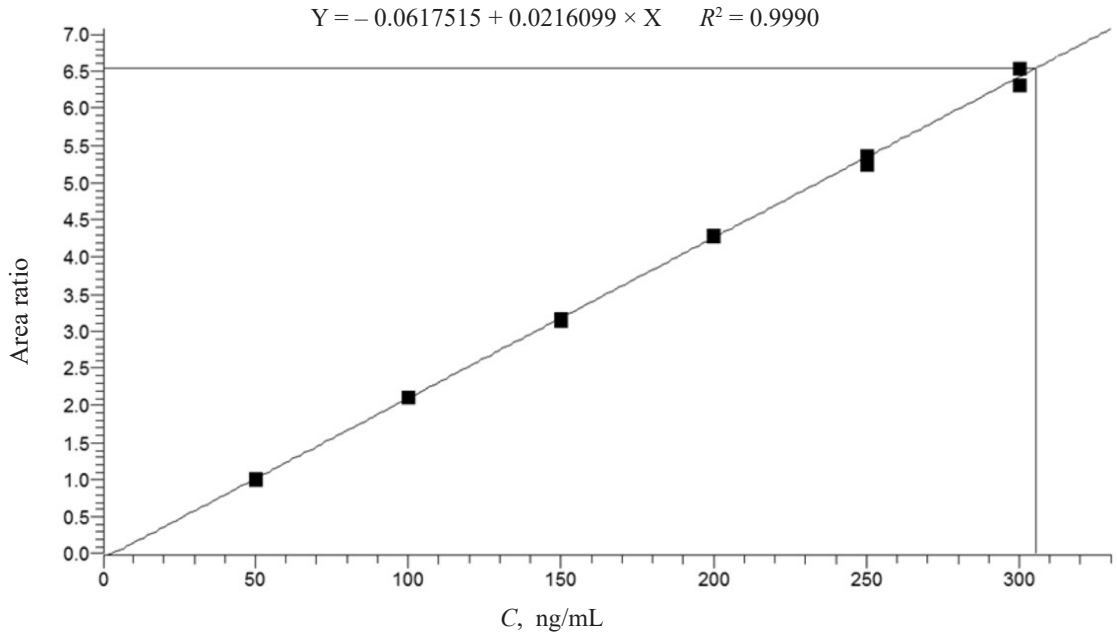

Figure. Linear calibration curve plot of hydromorphone.

aliquoting the urine sample with automatic dispensers, and diluting the urine sample (during the preparation of solutions).

The combined uncertainty value was calculated by formula (3). The maximum uncertainty value was 19%, the data are presented in Table 3. This method can be used to quantify the presented list of substances (see Table 1).

CONCLUSIONS

A new approach for the quantitative determination of 31 potent and narcotic substances and their metabolites in urine intended for introduction into the routine practice of NADL MSU was significantly revised and validated using a fast and highly sensitive UHPLC–MS/MS method.

The important advantages of the technique are the absence of complex and lengthy sample preparation (e.g., SPE and the formation of TMS derivatives), as well as a short analysis time of about 10 min. This allows the duration of the determination to be significantly reduced, along with labor and analysis costs. The addition of new detectable compounds will ensure the adopted method's versatility and allow its scope to be expanded without loss of sensitivity and selectivity when performing chemico-toxicological analysis or doping control.

The improved methodology has been revalidated in accordance with the requirements of ISO/IEC 17025-2019 and included in the scope of NADL MSU accreditation. Since the introduction of the validated methodology, more than 750 urine samples have been analyzed and more than 30 confirmed positive samples have been identified, which confirms the high level of detectability and sensitivity.

Authors' contributions

N.B. Savelieva – development of a plan for conducting experiments, analysis of the results obtained, primary processing of experimental data, and writing the text of the article;

G.V. Ishutenko – conducting experimental research, scientific and technical support, analysis of the obtained results, primary processing of experimental data, and editing the final version of the article;

A.V. Polosin – conducting experimental studies, scientific and technical support, analysis of the results obtained, and editing the final version of the article;

F.V. Radus – primary processing of experimental data;

D.S. Polyansky – verification and systematization of data validation parameters;

S.A. Kurbatkin – primary processing of experimental data;

Yu.A. Efimova – systematization and processing of the obtained results, editing the manuscript, and preparation of materials for publication;

P.V. Postnikov – formulation of aims and objectives, discussion of experiments and results, general management of the validation process, writing the text of the article, editing the manuscript, editing the final version of the article, and preparing materials for publication.

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