

Determination of 15 Functional State Biomarkers in Human Urine by High-Performance Liquid Chromatography with Tandem Mass Spectrometric Detection

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Abstract—The capabilities and limitations of complex procedures for targeted metabolomic analysis using liquid chromatography in combination with tandem mass spectrometry (HPLC-MS/MS) are discussed. An HPLC-MS/MS procedure for the simultaneous determination of the concentrations of 15 biomarkers of the functional state of a human being in urine is presented. The target analytes are biogenic substances of various chemical natures, the basic concentrations of which in biomatrices can vary significantly, up to three orders of magnitude or more. Complex analysis is also difficult to perform due to significant differences in the hydrophilic and hydrophobic properties of the analytes. The testing of the procedure in a bioanalytical experiment made it possible to establish significant differences in the concentrations of a number of biomarkers in the urine of persons with different levels of physical fitness. With a high level of physical fitness, the concentrations of these compounds in urine have lower values in comparison with those in the control group.

Keywords: liquid chromatography, mass spectrometric detection, metabolomics, functional state biomarkers, urinalysis

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Mass spectrometric methods are increasingly used in biomedicine, especially for early diagnosis and monitoring of diseases. Bioanalytical technologies, which use noninvasively sampled diagnostic biomedica as test materials transported from rural health posts in remote areas to specialized laboratories, are developing most actively. Functional diagnostics is the most important application of analytical mass spectrometry in medicine. Functional diagnostics using instrumental methods makes it possible to determine the cause of the patient's poor health, especially in cases where the symptoms do not give a clear clinical picture. With the development of modern technologies, it becomes possible to detect failures in the regulatory processes of the body more and more early. Analytical mass spectrometry solves many problems of functional diagnostics. The capabilities of chromatography–mass spectrometry are implemented in functional diagnostics mainly within the framework of metabolomics [1].

According to optimistic forecasts, functional metabolomics will be able in the foreseeable future to change from diagnostics to metabolome reprogramming [2]. However, the current development of the metabolomic approach in functional diagnostics is hampered not so much by the pace of a search for reliable functional state biomarkers but by the lack of reli-

able high-performance analytical methods for their determination.

The metabolomics of stress. The influence of stress on the functional state of the body has been studied from ancient times to the present day. Stress can be both a provoking and exacerbating factor for many diseases and pathological conditions, including insulin resistance [3], metabolic syndrome [4], cardiovascular diseases [5], etc. In most cases, metabolic responses to stress cannot be accurately predicted and should be diagnosed because they are extensive, multifaceted, and simultaneously individual [6]. Metabolic profiles of tissues and biofluids reflect functional changes in the body. The determination of metabolomic parameters of biofluids allows one not only to identify the body's response to stress and evaluate changes in the physiological status but also to monitor the effectiveness of pharmacological support under conditions of stressful effects or their consequences.

A multifaceted metabolic response to stress includes processes such as amino acid and protein metabolism disorders, lipid and carbohydrate metabolism disorders, mechanical and/or chemical damage to myocytes, and many others. The profile of low-molecular-weight metabolites in biofluids is formed as a result of a set of biochemical processes. It is

extremely difficult to establish key marker compounds that would be indicators of stressful impacts. Paradoxically, the difficulty is due to a huge amount rather than a lack of scientific data. Numerous studies performed by means of non-targeted metabolomics have identified many signaling biomarkers in relation to specific exposures or diseases. Effects due to even insignificant unidirectional changes in the concentrations of metabolites in the test biological media are significant if studies are carried out with the involvement of large data arrays. The question inevitably arises: can these minor changes be considered significant given the extremely low accuracy of determining the concentrations of biomarkers in non-targeted metabolomics. On the other hand, the same biomarkers often characterize the metabolic consequences of human body exposure to various stress factors, including physical overstrain [7, 8]. This circumstance makes it possible to identify a group of stress markers that are not associated with a particular type of stress. It can be assumed that the concentration of stress markers in diagnostic biofluids is the lower, the higher the resource of the body's resistance to stress. However, this hypothesis needs to be tested. The most promising direction is the study of the urinary signature of the physiological state of a person, which is formed as a result of determining the absolute concentrations or concentrations normalized to a certain indicator of a limited set of biogenic substances—stress markers.

Rationale for the choice of urine as an optimal biomatrix. In recent years, the metabolomic studies of urine formed urinomics as an independent scientific direction. Nutraceutical markers and markers of water balance, muscle status, etc., are examined by means of urinomics. The most important in this series are markers of tolerance to stress loads, that is, stress markers. Urine sampling is non-invasive; it can be carried out without the participation of medical staff, and it does not require sterility. Unlike blood plasma, urine does not require special treatment immediately after sampling, and the composition of organic compounds in urine is less susceptible to distortion in the course of sampling, storage, and transportation. As a rule, the concentrations of biogenic analytes in urine do not change significantly upon several freeze–thaw cycles. In comparison with blood, urine is less saturated with organic compounds that can be oxidized or act as promoters of oxidation processes. Bernini et al. [9] reported that metabolite levels in urine samples were stable for at least 24 h at 10°C and four weeks at 4°C. These periods of stability for blood plasma and, especially, for serum are much shorter.

Urine, unlike blood, does not have mechanisms to maintain homeostasis; therefore, the metabolic profiles of urine are more variable, and they respond more quickly to stress.

Targeted metabolomics of urine. In targeted metabolomics, achieving low limits of analyte detection is not always a priority because biogenic analytes (metabolites) are present in samples mainly at high concentrations. The most stringent requirements in targeted metabolomics are imposed on the reliability of quantitative determinations. Currently, there is no consensus on the optimal way to normalize the concentrations of biomarkers in the urine. Normalization is necessary because the water content of urine depends on water consumption and a number of other physiological factors. Normalization with reference to such indicators as creatinine concentration, density, osmolality, and probabilistic or integral normalization is used most frequently [10]. After performing a non-targeted metabolomic analysis using HPLC-MS/MS as the analytical method, Rosen-Wolmar et al. [11] compared the effectiveness of three common approaches for adjusting urinary metabolite concentrations—creatinine normalization, specific gravity (density) normalization, and probabilistic quotient normalization (PQN). The results showed that creatinine normalization is not a reliable approach to correct urinary metabolomics data. Density normalization and PQN have been characterized as more reliable approaches. It is most likely that the reason for the unreliability of the normalization of data from metabolomic studies for creatinine is the fact that creatinine itself is a sensitive biomarker involved in the implementation of responses to external influences.

The development, validation, and practical implementation of procedures for monitoring the physiological status of a human being provide for the solution of the following tasks:

- substantiation of a group of diagnostic markers determined in biosamples, which characterize the processes of lipolysis, glycolysis, general energy metabolism, neurotransmission, and metabolism of purines, choline, and creatine;
- development of an analytical strategy for the determination of a group of relevant stress markers in a biosample;
- determination of background concentrations of selected biomarkers in the test biosample (urine) and an approximate assessment of variations in background concentrations due to the influence of stress factors;
- determination of concentration ranges within which it is necessary to perform measurements for each analyte biomarker and the construction of calibration characteristics within the established ranges.

To date, acceptable accuracy in the determination of concentrations of biogenic substances in biosamples can be achieved only in the targeted analysis mode. According to published data [12, 13], preference in the determination of organic acids in urine should be given to gas chromatography with mass spectrometric detection, which makes it possible to

achieve efficient separation of components and operate in the region of low matrix effects. These procedures are more time-consuming and labor-intensive compared to HPLC-MS/MS because they include derivatization in addition to extraction.

As a rule, HPLC-MS/MS is a method of choice for the determination of analytes represented by a group of substances in a wide range of acid–base properties. In the dilute and shoot technique [14], the extraction of analytes from biomatrices is not performed in sample preparation for HPLC-MS/MS analysis; therefore, there is no need to sequentially extract acidic and basic analytes by varying pH. If the limiting error of analysis is set at a level of 25–30%, it is still possible to determine a limited number of compounds characterized by different chemical nature and different levels of background concentrations within a HPLC-MS/MS procedure. From the 1980s to the present, the targeted metabolomics of urine has been dominated by studies devoted to such an important topic as the diagnosis of congenital metabolic disorders. As new information about the biomarkers of congenital diseases becomes available, there is a need for procedures that cover the determination of an increasing number of analytes. Körper-Keularts et al. [15] described the determination of 71 analytes in urine by HPLC-MS/MS with a time-of-flight detector. As judged from the mass chromatograms presented in the supplementary materials to the cited article, the complete chromatographic separation of analytes could not be achieved. Moreover, chromatographic peaks of correct shapes were not obtained for most analytes; however, the procedure was validated with acceptable metrological characteristics.

Zheng et al. [16] proposed a procedure for the determination of 142 urinary metabolites belonging to different classes of compounds. Of the 142 metabolites, 67 were quantified, and the rest were determined semiquantitatively. In contrast to the targeted metabolomics of hereditary diseases, the metabolomics of stress is much less understood, and the formation of a metabolic signature of stress is a matter of the future, even though the biochemical basis of stress is deeply and comprehensively studied.

Formation of a group of target analytes. Forming a set of stress markers determined in urine, we focused on the works performed in a mode of untargeted metabolomics with the use of not only urine but also blood as a biomatrix. In this case, we proceeded from the fact that unconjugated polar analytes are rapidly filtered through the kidneys and excreted in the urine. Taking into account current concepts of the biochemistry of stress [17], we formed the following group of relevant biomarkers.

(1) secondary metabolic products of adenosine triphosphoric acid—inosine and hypoxanthine as markers of cardiovascular risks [18, 19];

(2) acetylcarnitine, adenosine, and creatine as markers of energy metabolism and neurotransmission [20, 21];

(3) 3-methylhistidine and 2-hydroxy-2-methylbutyrate as markers of myofibrillar proteolysis and the risk of muscle loss [22, 23];

(4) threonine as a marker of catabolic processes [24];

(5) tryptamine (a product of microbial biotransformation of tryptophan) as a marker of the body's immune status [25, 26];

(6) 3-hydroxybutyrate, 3-hydroxy-3-methylbutyrate and 2-hydroxybutyrate as markers of fatty acid and ketoamino acid catabolic pathways [27];

(7) lactic acid as a marker of hypoxia [28, 29];

(8) uridine as a marker of neurodegenerative processes [30];

(9) creatinine as a marker of dysfunction of the excretory system [31].

For the selected biomarkers, there is no possibility of an unambiguous interpretation of urinary concentrations. Systematic data on the concentrations of biomarkers in urine, both in the norm and in cases of deviations from the norm in violation of regulatory processes in the body, are almost absent. Few published data on the concentration levels of selected biomarkers in urine are given in relation to creatinine, the boundaries of a normal level of which in urine cover a whole order of magnitude, and this fact does not allow us to even approximately convert relative concentrations into absolute ones. In addition, creatinine itself is included in the group of selected biomarkers (Table 1).

Good exercise tolerance, which can be interpreted to a certain extent as stress resistance, is a feature of the physiological status of humans who are in good physical shape [32].

The aim of this work was to develop a procedure for the determination of 15 biogenic substances in human urine by high performance liquid chromatography with tandem mass spectrometric detection (HPLC-MS/MS) and to test this procedure in a bioanalytical experiment.

The procedure developed for the determination of stress markers in urine was tested in the analysis of urine samples of young (20–35 years old) persons with high and insufficient physical fitness.

EXPERIMENTAL

Reagents. We used acetonitrile for high performance liquid chromatography (Panreac, Spain), methanol of HPLC grade (J.T. Baker, the Netherlands), ammonium formate (Acros Organics, Belgium), creatinine, creatine, lactic acid, inosine, acetylcarnitine, threonine, 2-hydroxybutyrate, 3-hydroxybutyrate, 2-hydroxymethylbutyrate, 3-hydroxymethylbutyrate, hypoxanthine, 3-methylhisti-

Table 1. List of target biomarkers for monitoring in urine samples and their empirical formulas, hydrophobicity constants ($\log P$), molar weights, and structural formulas

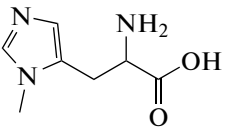
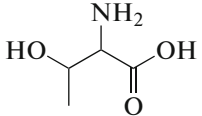
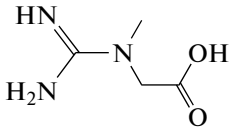
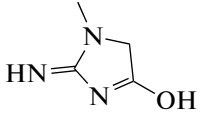
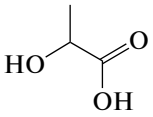
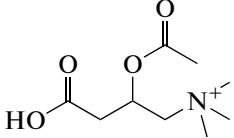
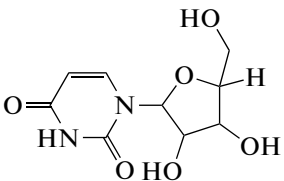
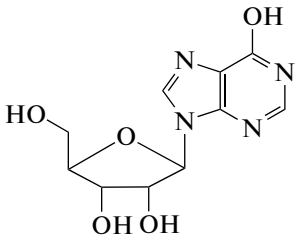
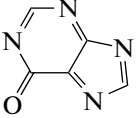
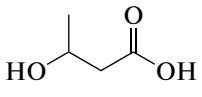
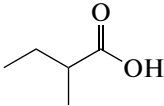
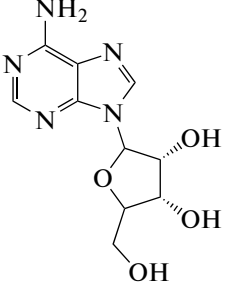
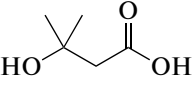
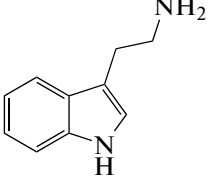
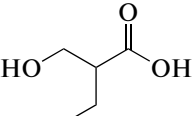
Biomarker	Empirical formula	$\log P^*$	Molar weight, g/mol	Structural formula
3-Methylhistidine	$C_7H_{11}N_3O_2$	-1.31	169.18	
Threonine	$C_4H_9NO_3$	-1.23	119.12	
Creatine	$C_4H_9N_3O_2$	-1.88	131.13	
Creatinine	$C_4H_7N_3O$	-1.68	113.12	
Lactic acid	$C_3H_6O_3$	-0.70	90.08	
Acetylcarnitine	$C_9H_{17}NO_4$	-3.56	203.24	
Uridine	$C_9H_{12}N_2O_6$	-1.61	244.20	
Inosine	$C_{10}H_{12}N_4O_5$	-1.45	268.23	
Hypoxanthine	$C_5H_4N_4O$	-2.51	136.11	
3-Hydroxybutyrate	$C_4H_8O_3$	-1.14	104.10	

Table 1. (Contd.)

Biomarker	Empirical formula	log <i>P</i> *	Molar weight, g/mol	Structural formula
2-Hydroxybutyrate	C ₄ H ₈ O ₃	-0.17	104.10	
Adenosine	C ₁₀ H ₁₃ N ₅ O ₄	-1.02	267.24	
3-Hydroxymethylbutyrate	C ₅ H ₁₀ O ₃	-0.79	118.13	
Tryptamine	C ₁₀ H ₁₂ N ₂	1.38	160.22	
2-Hydroxymethylbutyrate	C ₅ H ₁₀ O ₃	0.18	118.13	

The hydrophobicity constants (log *P*) were taken from the website <http://www.chemspider.com/> (Accessed March 28, 2023).

dine, adenosine, and uridine (Sigma-Aldrich, the United States). Deuterated (D-3) 2-(2-carboxyethyl)-1,1,1-trimethylhydrazinium (99.9% isotopic purity) was synthesized in the Chemical Modeling Laboratory of the Research Institute of Hygiene, Occupational Pathology, and Human Ecology according to a procedure described by Gørgens et al. [33].

Preparation of calibration solutions. Stock solutions of 2-hydroxybutyrate, 3-hydroxybutyrate, 2-hydroxymethylbutyrate, 3-hydroxymethylbutyrate, lactic acid, creatinine, uridine, inosine, creatine, 3-methylhistidine, adenosine, threonine, and acetylcarnitine were prepared by dissolving weighed portions of substances in deionized water. For the complete dissolution of a sample of hypoxanthine in deionized water, the solution was made alkaline by adding dropwise a 5% aqueous solution of ammonia. Tryptamine was dissolved in acetonitrile.

Sampling and sample preparation of urine. Fasted samples were taken in the morning, provided there was no intense physical activity during the previous day. Urine samples were frozen and stored at -18°C before the analysis.

Deuterated (D-3) 2-(2-carboxyethyl)-1,1,1-trimethylhydrazinium was chosen as an internal standard. The use of an isotope-labeled standard made it possible to eliminate the preliminary control of the test urine samples for the presence of background signals in the area of detection of a peak of the internal standard.

Sample preparation was carried out as follows: 0.3 mL of a urine sample and 0.9 mL of acetonitrile containing an internal standard with a concentration of 3 µg/mL were added to a 2.0-mL plastic tube; the contents were thoroughly mixed and centrifuged at a speed of 14000 rpm for 5 min. The supernatant was diluted with a 0.1% solution of formic acid in deionized water by factors of 10 and 1000 times and analyzed by HPLC-MS/MS. Creatinine, uridine, and inosine were determined in the samples diluted by a factor of 10.

HPLC-MS/MS analysis. Urine samples were analyzed using an LC-20 Prominence liquid chromatograph equipped with an LCMS-8050 mass spectrometric detector with electrospray ionization at atmospheric pressure (Shimadzu, Japan). Data were

Table 2. Analytical characteristics of the test biomarkers: ionization mode, retention time (Rt), MPM transition (precursor ion > product ion), and collision energy (CE)

Biomarker (ionization mode)	Rt, min	MPM transition, m/z (CE)
3-Methylhistidine (+)	3.83	170.10 > 109.15 (−16); 126.20 (−16)
Threonine (+)	4.04	120.00 > 74.10 (−12); 56.10 (−17)
Creatine (+)	4.47	132.00 > 44.10 (−14); 90.05 (−22)
Creatinine (+)	4.56	114.00 > 44.1 (−17); 86.1 (−14)
Lactic acid (−)	6.13	89.00 > 43.1 (12); 45.05 (12)
Acetylcarnitine (+)	6.49	204.00 > 85.1 (−21); 145.15 (−13)
Uridine (+)	7.83	245.00 > 113.10 (−12); 70.10 (−35)
Inosine (+)	8.15	269.00 > 137.10 (−11); 110.10 (−23)
Hypoxanthine (−)	8.24	135.00 > 92.00 (18); 65.00 (25)
3-Hydroxybutyrate (−)	8.24	103.00 > 59.00 (11); 45.00 (25)
2-Hydroxybutyrate (−)	8.63	103.00 > 57.05 (14)
Adenosine (+)	8.93	268.00 > 136.10 (−22); 119.10 (−44)
3-Hydroxymethylbutyrate (−)	9.29	117.00 > 59.10 (12); 41.20 (25)
Tryptamine (+)	9.60	161.00 > 144.15 (−14); 117.15 (−25)
2-Hydroxymethylbutyrate (−)	9.96	117.00 > 71.15 (13)
Deuterated (D-3) 2-(2-carboxyethyl)-1,1,1-trimethylhydrazinium internal standard (+)	4.84	150.00 > 61.15 (−16); 62.15 (−16)

processed using Quant Browser in the Labsolution software (Shimadzu, Japan).

Chromatographic separation was carried out on a Zorbax SB-C8 column (Agilent, the United States) (150 × 4.6 mm, 1.8 μm). Mobile phase: component A, 0.1% formic acid and a 10 mM solution of ammonium formate in deionized water; component B, 0.1% formic acid and a 10 mM solution of ammonium formate in methanol. Elution program at an eluent flow rate of 0.4 mL/min: 0.0–1.0 min, 5% B; 1.0–7.0 min, 5–90% B; 7.0–10.0 min, 90% B; 10.1–15.0 min, 5% B. Column oven temperature was 40°C. Sample compartment temperature was 5°C. The injected sample volume was 5 mL.

Mass spectrometric detection was carried out in the multiple reaction monitoring (MRM) mode based on the specified ion reactions (MRM transitions) precursor ion > product ion (Table 2).

Ionization source parameters: dryer-gas flow rate, 10 L/min; auxiliary gas flow rate, 10 L/min; spray pressure, 3 L/min; dryer gas temperature, 300°C; auxiliary flow temperature, 350°C; capillary voltage, 3500 V; and fragmentator voltage, 120 V.

Preliminary optimization of the detection parameters (m/z of precursor and product ions and collision energy) was performed automatically using the Auto-tuning setting.

Statistical processing of measurement results. Differences between the concentrations of biomarkers in the urine samples of two groups of volunteers were assessed using the Welch parametric *t*-test after the logarithmic transformation of experimental data. The results were considered statistically significant at $p < 0.05$. All calculations were performed in GraphPad Prism 8.

Male and female humans aged 20–35 years took part in the bioanalytical study to test the procedure. Volunteers were divided into two groups of low trained (LT) and high trained (HT) persons. The LT group ($n = 9$) was represented by people leading an inactive lifestyle: office work at the computer, irregular physical activity, and a body mass index of >30. The HT group ($n = 29$) included people who lead an active lifestyle, visit the gym at least twice a week, and have a body mass index of 20–27. Informed consent was

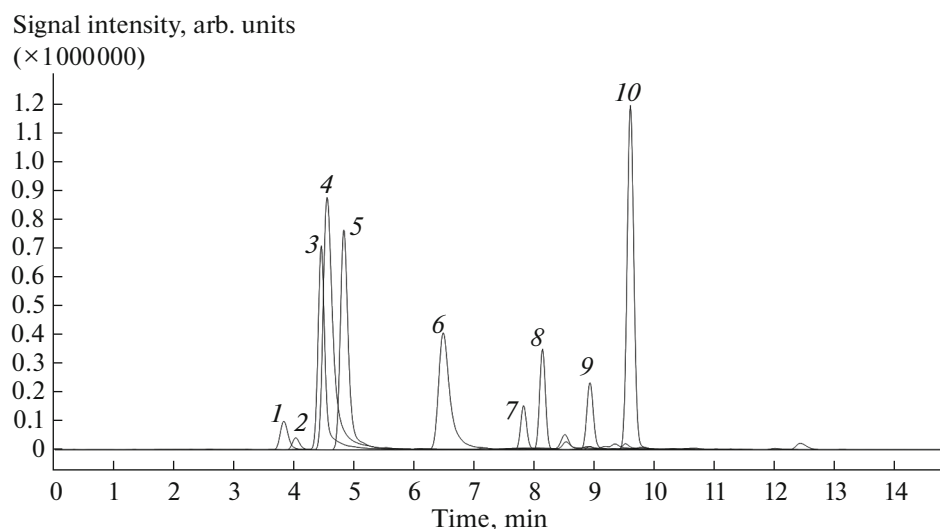


Fig. 1. Mass chromatogram in the positive ionization mode: (1) 3-methylhistidine, (2) threonine, (3) creatinine, (4) creatinine, (5) deuterated (D-3) 2-(2-carboxyethyl)-1,1,1-trimethylhydrazinium, (6) acetylcarnitine, (7) uridine, (8) inosine, (9) adenosine, and (10) tryptamine.

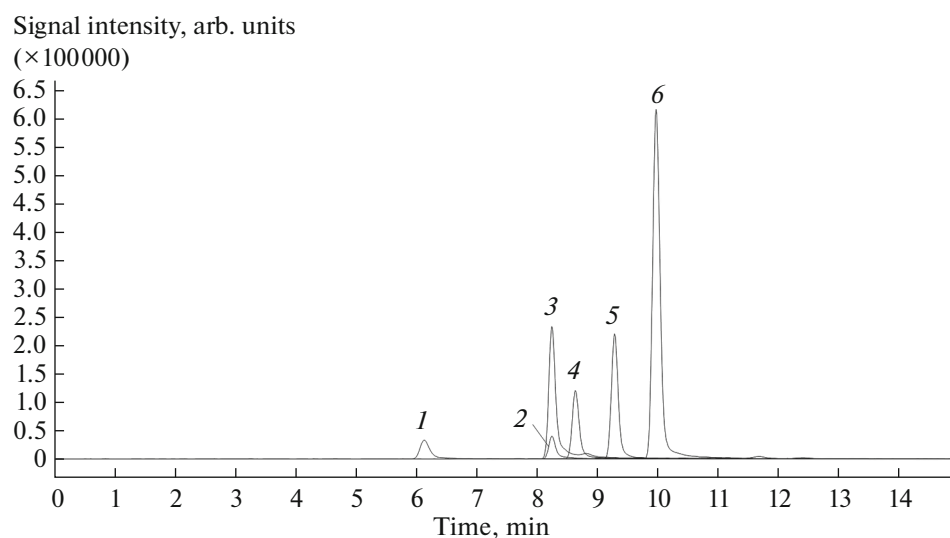


Fig. 2. Mass chromatogram in the negative ionization mode: (1) lactic acid, (2) hypoxanthine, (3) 3-hydroxybutyrate, (4) 2-hydroxybutyrate, (5) 3-hydroxymethylbutyrate, and (6) 2-hydroxymethylbutyrate.

obtained from the volunteers to participate in the experiment.

RESULTS AND DISCUSSION

HPLC-MS/MS analysis procedure. Figures 1 and 2 show the chromatograms of the test substances. Table 1 indicates that the hydrophilic and hydrophobic properties of the analytes varied over a wide range, which should be taken into account when choosing the mode of chromatographic separation. As can be seen in the presented mass chromatograms, the selected

elution mode made it possible to achieve an acceptable separation of all analytes.

The metrological characteristics of the proposed procedure for the joint determination of 15 biomarkers of the functional state of humans were obtained based on the determination of their concentrations in aqueous solutions and taking into account the range of levels of their concentrations in the urine of volunteers (Table 3).

The preparation of samples for analysis was limited to dilution followed by centrifugation; in this case, high recovery rates of analytes were achieved: from 95.2 to 98.9%.

Table 3. Metrological characteristics of the proposed procedure

Biomarker	Analytical range, $\mu\text{g/mL}$	Reproducibility RSD, %	Accuracy (limits of relative systematic error at $P = 0.95$), $\pm\delta_c$, %	Precision (limits of relative systematic error at $P = 0.95$), $\pm\delta$, %
3-Methylhistidine	1–50	1.0	3.0	4.8
Threonine	1–50	7.0	1.3	7.1
Creatine	1–50	10.6	1.4	8.8
Creatinine	0.2–10	6.4	2.6	6.6
Lactic acid	2–100	7.7	1.4	8.1
Acetylcarnitine	2–100	6.7	0.9	7.2
Uridine	0.2–10	9.1	2.1	7.5
Inosine	0.2–10	4.0	1.2	5.2
Hypoxanthine	1–50	11.3	1.1	10.3
3-Hydroxybutyrate	1–50	5.7	1.0	6.7
2-Hydroxybutyrate	1–50	4.8	1.3	5.4
Adenosine	1–50	6.0	1.4	6.5
3-Hydroxymethylbutyrate	2–100	5.6	1.0	6.5
Tryptamine	1–50	7.2	1.8	8.1
2-Hydroxymethylbutyrate	1–50	3.8	2.2	5.5

Comparison of the results of the determination of stress marker concentrations in the urine of volunteers from the HT and LT groups. Before the statistical processing, the results were tested for normal/lognormal distributions. Due to the small number of observations in the LT group, the distributions were lognormal for all biomarkers other than creatinine. For creatinine, a normal distribution was observed. For this reason, the application of the parametric criterion was possible after the logarithmic transformation of the results [34], which was performed according to the formula

$$y = \log(x + 0.01), \quad (1)$$

where y is the converted value of the found biomarker concentration; x is the found value of the biomarker concentration; 0.01 is a constant required to correct calculations in the presence of results below the lower limit of determined concentrations.

The data obtained after the logarithmic transformation were evaluated using the Welch t -test. The arithmetic mean, standard deviation, and confidence interval were calculated. Then, the molar concentrations were back-calculated. For example, the arithmetic mean of data for lactic acid in the HT group after logarithmic transformation was 2.226, and the confidence interval (at $p = 0.95$) was 0.080. In accordance with an approach proposed by West [34], the reference range for lactic acid was calculated as follows: $(2.226 \pm [1.96 \times 0.080])$ or $(2.070–2.382)$. Upon the inverse transformation, it was $(10^{2.070}–10^{2.382})$, which corresponds to $(117.6–241.2) \mu\text{M}$. Other data were obtained in a similar manner, with the exception of creatinine

for which logarithmic transformation was not performed.

To evaluate the concentrations of biomarkers normalized to creatinine, their levels were first calculated relative to creatinine ($\mu\text{mol}/\text{mmol}$ creatinine), and then a logarithmic transformation and analysis of the obtained data were performed with further recalculation according to the algorithm described above.

Table 4 summarizes the results of measurements (reference range) converted from weight to molar concentrations taking into account the molar weights of analytes (Table 1) and the subsequent normalization to creatinine.

The normalization of urinary biomarkers to creatinine is usually based on the assumption that the rate of creatinine excretion with urine is constant for different persons and also on the fact that the concentration of substances dissolved in urine depends on the process of water reabsorption in the kidneys [35]. However, the normalization of creatinine data to assess the results of urine tests of people with abnormal kidney function [35, 36] leads to a distortion of their interpretation.

Normalization to the level of creatinine led to smoothing of the data due to relatively close values of creatinine in the urine because young people without diagnosed diseases took part in our experiment; therefore, data both with and without normalization to creatinine are given.

The results obtained in the testing of the procedure (Table 4) should be interpreted with caution. With all the previously noted advantages of urine as a bioma-

Table 4. Results of measurements (reference ranges) in the urine of volunteers

Biomarker	Reference range (without normalization to creatinine), μmol		Reference range (with normalization to creatinine), $\mu\text{mol}/(\text{mmol creatinine})$	
	HT ($n = 29$)	LT ($n = 9$)	HT ($n = 29$)	LT ($n = 9$)
Creatinine (mM)	4.0–4.8	4.7–6.9*		
3-Methylhistidine	21.5–47.0	29.1–92.2	5.6–11.3	5.5–16.0
Threonine	94.4–162.1	119.2–254.9	22.5–38.1	25.1–39.6
Creatine	80.0–177.5	79.8–290.9	19.9–40.0	14.8–51.4
Lactic acid	117.6–241.2	265.3–675.0	28.5–55.7	51.8–113.0**
Acetylcarnitine	177.2–463.9	115.9–1106.6	36.3–98.2	32.0–176.4
Uridine	5.4–10.1	4.5–12.5	1.8–2.9	1.7–2.6
Inosine	1.8–3.4	3.8–15.0	2.2–5.4	1.6–3.0
Hypoxanthine	97.8–142.1	84.4–245.3	23.8–32.7	17.8–38.0
3-Hydroxybutyrate	15.7–26.6	24.0–82.7*	3.8–5.9	4.1–16.1
2-Hydroxybutyrate	222.6–384.6	249.9–535.7	55.6–86.2	49.9–87.7
Adenosine	11.2–19.2	19.5–53.3	2.8–4.3	4.0–8.5*
3-Hydroxymethylbutyrate	173.9–314.1	371.2–971.1	43.7–70.0	69.4–169.9**
Tryptamine	34.5–41.6	38.0–56.4	8.0–10.0	6.8–10.3
2-Hydroxymethylbutyrate	28.2–48.0	34.3–74.6	7.0–10.8	6.7–12.5

Significant differences at: * $p < 0.05$ and ** $p < 0.01$.

trix, limitations in interpreting the results of urine metabolomics should also be taken into account. First of all, this is the influence of the diet on the metabolic profiles of urine. The volunteers who took part in the experiment, according to the questionnaire, did not adhere to a diet and did not take food supplements or drugs for a week preceding the test, while their diet was not standardized, and this fact could not but affect the test results. Nevertheless, the results obtained allowed us to note some trends and draw preliminary conclusions. Despite the fact that the LT group was less numerous ($n = 9$) compared to the HT group ($n = 29$), wider ranges of concentration values for most biomarkers can be noted for it; that is, the HT group can be characterized as more homogeneous. Another trend is a shift in the concentrations of most stress markers toward higher values in the urine of people from the LT group compared to those in the HT group. This result can be explained by the fact that the state of rest (there was no physical activity on the eve of urine sampling) in the HT group was characterized as complete relaxation, and this time was lived with a non-zero level of stress in the LT group. Another possible explanation is the reduced ability of LT volunteers to

reabsorb stress markers by the kidneys and/or distribute them to tissues.

Significant differences in the concentrations of stress markers in the urine of volunteers from the two groups were established for creatinine and 3-hydroxybutyrate without normalization to creatinine. Significant differences were also found for lactic acid, adenosine, and 3-hydroxymethylbutyrate both without and with normalization to creatinine.

The result obtained does not contradict published data indicating that lactic acid, hydroxybutyrates, and adenosine are included in the group of biomarkers that characterize the level of physical fitness, which is largely determined by exercise tolerance [37–39].

It should be noted that different biomarkers have been established to characterize the level of physical fitness in various sources [40, 41]. All of them still have the status of candidate biomarkers. A necessary step toward the formation of a list of relevant stress markers in various diagnostic biomedica is the development of procedures for the determination of candidate biomarkers.

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ETHICS APPROVAL

All procedures performed in this study that involved human participants comply with the ethical standards of the institutional and/or national research ethics committee and the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

INFORMED CONSENT

Informed consent was obtained from all participants included in the study.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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