The main step of bioequivalence and pharmaco-kinetic studies is a determination drug substance of concentration in biological fluids, such as plasma, serum and whole blood. Some substances are able to significantly decompose during the storage of samples. Are drugs, containing phenolic hydroxyls. Examples of the substances. The oxidation ability of phenols directly depends from the amount of phenolic hydroxyls in one benzene ring [2]. Mycophenolic acid (MPA) (Fig. 1 A) and methyldopa (MD) (Fig. 1 B) which contains one and two phenolic hydroxyls, respectively, were selected to work out approaches development of bioanalytical methods of the quantitative determination of drugs, containing phenolic hydroxyls.
Detection of mycophenolic acid was carried out in SIM negative ions mode by the molecular ion with 319 m/z.

Results and Discussion. The preliminary stability study of mycophenolic acid was carried out in plasma samples at the concentration of 25 μg/ml using different anticoagulants (K₃EDTA and heparin). The calculation of the MPA concentration at this step was performed using the external standard method. The results obtained after 24 hours storage of samples at room temperature and 3 freeze/thaw cycles meet the acceptance criteria: the mean MPA concentrations were 95.2 % and 102.1 % of the theoretical concentration, respectively. Therefore, this analyte is stable, and addition of antioxidant is not required. However, the main metabolite of MPA is the phenolic glucuronide (MPAG). Its plasma concentrations can reach 100 μg/ml [9]. The back conversion during storage of this compound has been insufficiently studied: a number of publications indicate the necessity of using the buffer solutions to prevent this phenomenon [5, 6]. Some studies indicate that hydrolysis of MPAG is not significant, and the addition of stabilizers is not required [4, 11]. However, back conversion of MPAG was not investigated in the majority of researches [7, 8, 13, 15, 20–22].

Aliquots of 50 μl of samples containing a metabolite at a concentration of 100 μg/ml, were taken at a certain storage time at room temperature, and the MPA peak areas of these samples were compared with the peak area of the MPA samples at the concentration of LLOQ level (Table 1) during evaluation of back conversion.

The level of back conversion was within the acceptable limits (less than 20 % of the peak area of the LLOQ samples) for 6 hours using anticoagulant K₃EDTA. It is much longer than in the heparin samples. Plasma stabilizing heparin can be stored at room temperature for no longer than 1 h. Therefore, plasma with the addition of K₃EDTA was used for validation tests.

The preliminary evaluation of short-term stability and freeze/thaw stability of methyl dopa was carried out on plasma samples at the concentration level of 2.40 μg/ml using K₃EDTA and heparin as anticoagulants by comparing the ratios of peak areas <analyte/internal standard> obtained before and after investigation. MD underwent significant oxidative degradation during application of both anticoagulants (Table 2). Therefore, addition of antioxidant is necessary to prevent oxidation of MD in plasma.

Material and Methods. The quantitative determination of methyl dopa was performed on a Shimadzu HPLC-MS/MS system equipped with two LC-20AD pumps, an SIL-20AC autosampler, a STO-20AC column thermostat with an integrated 6-port valve and a triple quadrupole mass spectrometer detector LCMS-8050.

Protein precipitation was used for sample preparation of plasma containing MD. An aliquot 400 μl of a deuterated internal standard MD (MD-D₃) methanol solution were added to 100 μl of plasma. The mixture was vortexed and centrifuged for 10 min at 3500 rpm and a temperature of +4 °C. The supernatant was injected into the chromatographic system. The separation of the sample components was performed using two chromatographic columns Phenomenex Luna Phenyl-Hexyl (50 × 3.0 mm, 5 μm) and Phenomenex Synergi Fusion RP 80 Å (150×3.0 mm, 4 μm). The mixture of methanol, water and an aqueous solution of ammonium formate in a concentration of 80 mmol/l were used as the mobile phase (40:40:20 v/v). Mass spectrometric detection was carried out in positive ion mode using electrospray ionization. The MRM-transitions 212→139 m/z was selected for MD; 215→169 m/z for MD-D₃.

The study of short-term stability and freeze/thaw stability of mycophenolic acid consisted of G1311C pump, the G1329B ALS autosampler, the G1316A column thermostat and the 6130 Single quadrupole mass spectrometer detector LCMS-8050. The Agilent 1260 Infinity HPLC-MS system for quantification of both substances [5–9, 11–17, 19–22]. There is potential risk of methyldopa oxidation due to two phenolic hydroxyls in the structure. It is known that 10 % sodium metabisulphite solution is required for stabilization of dopamine in human plasma [18]. Dopamine is similar to antioxidant solutions to biological fluids samples is not necessary for quantification of both substances [5–9, 11–17, 19–22]. However, the main metabolite of MPA is the phenolic glucuronide (MPAG). Its plasma concentrations can reach 100 μg/ml [9]. The back conversion during storage of this compound has been insufficiently studied: a number of publications indicate the necessity of using the buffer solutions to prevent this phenomenon [5, 6]. Some studies indicate that hydrolysis of MPAG is not significant, and the addition of stabilizers is not required [4, 11]. However, back conversion of MPAG was not investigated in the majority of researches [7, 8, 13, 15, 20–22].

Sample preparation was also performed using protein precipitation: an aliquot of 50 µl plasma was mixed and vortexed with 200 µl methanol. The mixture was centrifuged for 5 min at 10000 rpm. 5 µl of the supernatant was processed to a LC/MS determination. The chromatographic separation was performed using Agilent Zorbax Eclipse Plus C18 column (100×4.6 mm, 3.5 μm) with isocratic elution of the mobile phase composed of acetonitrile, water and 0.1 % solution of formic acid (50:45:5 v/v) at a flow rate 0.4 ml/min and oven temperature 40 °C.

The results of publication showed that addition of antioxidant solutions to biological fluids samples is not necessary for quantification of both substances [5–9, 11–17, 19–22]. There is potential risk of methyl dopa oxidation due to two phenolic hydroxyls in the structure. It is known that 10 % sodium metabisulphite solution is required for stabilization of dopamine in human plasma [18]. Dopamine is similar to antioxidant solutions to biological fluids samples is not necessary for quantification of both substances [5–9, 11–17, 19–22]. However, the main metabolite of MPA is the phenolic glucuronide (MPAG). Its plasma concentrations can reach 100 μg/ml [9]. The back conversion during storage of this compound has been insufficiently studied: a number of publications indicate the necessity of using the buffer solutions to prevent this phenomenon [5, 6]. Some studies indicate that hydrolysis of MPAG is not significant, and the addition of stabilizers is not required [4, 11]. However, back conversion of MPAG was not investigated in the majority of researches [7, 8, 13, 15, 20–22].

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### Table 1

| K₃EDTA | Mean peak area of LLOQ sample | Initial | 1 h | 2 h | 3 h | 4 h | 6 h | 8 h | 24 h |
|-------|-----------------------------|--------|----|----|----|----|----|----|-----|
| Peak area of MPA | 8073.5 | 339.6 | 485.3 | 520.4 | 1077.8 | 1374.7 | 1801.0 | 3632.7 |
| % of the peak area of LLOQ sample | - | 4.2 | 6.0 | 6.4 | 13.3 | 17.0 | 22.3 | 45.2 |

### Table 2

|  | Mean peak area of LLOQ sample | Initial | 1 h | 2 h | 3 h | 4 h | 6 h | 8 h | 24 h |
|---|-----------------------------|--------|----|----|----|----|----|----|-----|
| Peak area of MPA | Heparin | 6512.0 | 523.0 | 1204.2 | 1556.0 | 3568.5 | 4899.0 | 7851.4 | 10045.0 | 523.0 |
| % of the peak area of LLOQ sample | - | 18.5 | 23.9 | 54.8 | 75.2 | 120.6 | 154.3 | 8.0 |

The study of short-term stability and freeze/thaw stability of methyl dopa was carried out on plasma samples at the concentration level of 2.40 μg/ml using K₃EDTA and heparin as anticoagulants by comparing the ratios of peak areas <analyte/internal standard> obtained before and after investigation. MD underwent significant oxidative degradation during application of both anticoagulants (Table 2). Therefore, addition of antioxidant is necessary to prevent oxidation of MD in plasma.
The selection of stabilizers to prevent oxidation of methyldopa

| Stabilizer | Concentration of stabilizer, % | Short-term stability (24 h at room temperature), % of initial concentration n=2 | Freeze and thaw stability, % of initial concentration n=2 |
|------------|--------------------------------|--------------------------------------------------------------------------|------------------------------------------------------------------|
| K$_3$EDTA  |                                |                                                                          |                                                                  |
| Without stabilizer |                              | 30.29                                                                   | 80.04                                                              |
| Ascorbic acid | 5                              | 88.44                                                                   | 102.30                                                            |
| 10             | 94.91                                                                       | 96.17                                                               |
| A mixture of ascorbic acid, sodium bicarbonate, sodium sulphite | 5 0.2 % ascorbic acid, 0.2 % sodium sulphite, 2.4 % sodium hydrogencarbonate | 89.31                                                               | 98.24                                                             |
| Sodium thiosulfate | 5                              | 45.65                                                                   | 101.86                                                            |
| 10             | 31.32                                                                       | 99.81                                                               |
| Sodium metabisulfite | 5                              | 66.84                                                                   | 102.68                                                            |
| 10             | 75.99                                                                       | 97.25                                                               |
| Heparin       |                                |                                                                          |                                                                  |
| Without stabilizer |                              | 47.16                                                                   | 93.18                                                             |
| Ascorbic acid | 5                              | 80.91                                                                   | 99.69                                                             |
| 10             | 78.76                                                                       | 93.21                                                               |
| A mixture of ascorbic acid, sodium bicarbonate, sodium sulphite | 5 0.2 % ascorbic acid, 0.2 % sodium sulphite, 2.4 % sodium hydrogencarbonate | 72.48                                                               | 95.07                                                             |
| Sodium thiosulfate | 5                              | 79.41                                                                   | 93.91                                                             |
| 10             | 83.46                                                                       | 97.78                                                               |
| Sodium metabisulfite | 5                              | 75.79                                                                   | 90.70                                                             |
| 10             | 73.22                                                                       | 88.70                                                               |

The selection of stabilizer and anticoagulant combination was performed by adding aqueous solutions of ascorbic acid, sodium sulfite, thiosulfate and metabisulfite in concentrations of 5 and 10 %, as well as a mixture of ascorbic acid, sodium sulfite, sodium hydroxy carbonate in the ratio of 0.2 ml of antioxidant solution per 1 ml of plasma. The concentration was also estimated by comparison of the peak area ratios analyte/anticoagulant per 1 ml of plasma. The concentration was also estimated by comparison of the peak area ratios analyte/anticoagulant per 1 ml of plasma.

Methyldopa was stable for 24 h at room temperature, and also during 3 freeze-thaw cycles after usage of ascorbic acid solutions in a concentration of 5 and 10 %, as well as a mixture of ascorbic acid, sodium sulfite, sodium hydroxy carbonate in concentrations of 5 %, 0.2 % and 2.4 %, respectively, in combination with K$_3$EDTA. The study of more concentrated ratios (1:2 or 1:1) was not carried out because the desired effect was achieved by addition antioxidant solutions to the plasma at a ratio of 1:5.

The mixture of ascorbic acid, sodium sulfite and sodium hydrocarbonate was selected for further method validation, because the area of the chromatographic peak of MD after their addition to the plasma was the highest (7756996, n=6) in comparison with the peak areas after addition 5 and 10 % ascorbic acid solutions (6465608 and 6720010, respectively, n=6) to the plasma.

Deproteinizeats of plasma samples containing solutions of sodium sulfite in methanol were converted into a viscous gel-like mass after 2 h of storage under autosampler conditions. It is prevented their injection into a chromatographic system. Therefore, the usage of this antioxidant is impossible. But the methanol deproteinizeats retained its rheological properties after using sodium sulfite in low concentrations in combination with ascorbic acid and sodium hydrocarbonate (Fig. 2, 3).

The validation of the developed methods was conducted in accordance with the requirements of EMA Guideline [10], Guideline on the Evaluation of Medicinal Products [3], Decision of the Council of the Eurasian Economic Commission No 85 «On the Approval of the Rules for Conducting of Bioequivalence Studies on the of Medicinal Products in the Eurasian Economic Union» [11]. Validation tests for MD were performed with the addition of a solution of stabilizer to blank plasma. The results of validation tests meet to acceptance criteria (Table 3).

| Method                              | Methyldopa (HPLC-MS/MS) | Mycophenolic acid (HPLC-MS) |
|-------------------------------------|-------------------------|----------------------------|
| Parameter                           | Results                 | Results                    |
|                                     |                         |                            |
| Selectivity                         | The interference in the area of retention times of MD did not exceed 20 % of the LLOQ level, and the interference in the retention times of MD-D3 did not exceed 5 % of the mean chromatographic peak area | The chromatograms did not have any interference at the retention time of MPA |
| Lower limit of quantification (LLOQ) | 0.02 μg/ml (relative error +4.20 %, precision (CV*) –1.59 %) | 0.05 μg/ml (relative error –3.73 %, precision (CV) –1.59 %) |
| Linearity                           | The concentration range: 0.05–30.00 μg/ml. The correlation coefficient of the 8-point calibration curve (r) was ranged from 0.9977 to 0.9993 | The concentration range: 0.05–30.00 μg/ml. The correlation coefficient of the 8-point calibration curve (r) was ranged from 0.9992 to 0.9995 |
| Precision and accuracy              | Relative error was ranged from –5.28 % to +7.42 %; CV was ranged from 0.69 % to 3.97 % | Relative error was ranged from –14.02 % to +11.82 %; CV was ranged from 0.37 % to 6.76 % |
| Recovery                            | 63.49 %                 | 80.82 %                    |
| Dilution integrity                  | Relative error: +0.21 % (n=6), CV=1.78 % (Twofold dilution of samples with analyte concentration of 4.80 μg/ml) | Relative error: +3.82 % (n=6), CV=6.27 % (Twofold dilution of samples with analyte concentration of 50.00 μg/ml) |
| Matrix effects                      | NMF** was ranged from 1.010 to 1.018; CV was ranged from 0.40 to 1.27 % | MFF** was ranged from 0.789 to 0.808; CV was ranged from 2.96 to 5.60 % |
| Short-term stability (24 h)         | 95.50 % of the theoretical concentration | 99.46 % of the theoretical concentration |
| Long-term stability (1 month)       | 90.74 % of the theoretical concentration | 100.38 % of the theoretical concentration |
| Freeze and thaw stability           | 104.43 % of the theoretical concentration | 103.55 % of the theoretical concentration |

The note: *CV – coefficient of variation; **NMF – normalized matrix factor; ***MF – matrix factor.
Fig. 2. The chromatograms of blank plasma (A) and plasma with methyldopa in the concentration of 0.02 µg/ml (LLOQ) (B)

Fig. 3. The chromatograms of blank plasma (A) and plasma with MPA in the concentration of 0.05 µg/ml (LLOQ) (B) and 30.00 µg/ml (C)

Conclusions. Thus, development the method for bioassay of potentially unstable compounds, such as phenolic substances, need to be started with the analysis and evaluation of structural features base on literature data. It is also necessary to pay attention information about the drug and similar structure substances. The selection of storage conditions should begin, with selection of anticoagulant based on the study of short-term stability and freeze/thaw stability. If an unsatisfactory result was obtained, the combination of anticoagulant and antioxidant solution, the concentration of the solution and volume ratio "biological fluid/antioxidant solution" should be investigated. The validation of the method should be started using anticoagulant and antioxidant after the selection (Fig. 4).
The method of quantitative determination of MD in plasma was applied in the bioequivalence study of the tablet form in a dosage of 200 mg. The method of quantification of mycophenolic acid was used for pharmacokinetic study of sodium mycophenolate in rats, and also was verified using the previously published HPLC-MS/MS method.

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**About authors:**

Khokhlov Alexander Leonidovich, DMSc, Professor, Corresponding Member of The Russian Scientific Academy; Head of the Department of Clinical Pharmacology; tel.: +79106631155; e-mail: ale460935@yandex.ru

Yaichkov Ilya Nikolaevich, CBSc, Head of bioanalytical laboratory, Assistant of the Department of Polyclinical Therapy and Clinical Laboratory Diagnostic; tel.: +79106631155; e-mail: schitov@inbox.ru

Dzhurko Yuriy Alexandrovich, CPhSc, Senior analyst; tel.: +79109759248; e-mail: y.dzhurko@qayar.ru

Yaichkov Ilya Igorevich, Post-graduate student of the Department of Clinical Pharmacology; tel.: +79106631155; e-mail: al460935@yandex.ru

Khokhlov Alexander Leonidovich, DMSc, Professor, Corresponding Member of The Russian Scientific Academy; Head of the Department of Clinical Pharmacology; tel.: +79106631155; e-mail: ale460935@yandex.ru

Yaichkov Ilya Nikolaevich, CBSc, Head of bioanalytical laboratory, Assistant of the Department of Polyclinical Therapy and Clinical Laboratory Diagnostic; tel.: +79106631155; e-mail: schitov@inbox.ru

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**INTERRELATION OF ENDOTHelial NITRic oxide SYNTHase ACTIVITY IN TISSUES OF THE STOMACH AND MAGNESIUM BALANCE IN THE PERIOD OF EROsive-ULCERATIVE ACID-INDUCED LesION DEVELopMENT IN RATS WITH DIFFERENT RESISTANCE TO STRESS**

**Rogova L. N., Povetkina V. N.**

**Volgograd State Medical University, Russian Federation**

**ВЗАИМОСВЯЗЬ АКТИВНОСТИ ЭНДОТЕЛИАЛЬНОЙ НИТРОКСИДСИНТАЗЫ В ТКАНЯХ ЖЕЛУДКА И МАГНИЕВОГО БАЛАНСА В ПЕРИОД ФОРМИРОВАНИЯ ЭРОЗИВНО-ЯЗВЕННОГО ДЕФЕКТА АЦЕТАТНОЙ ПРИРОДЫ У КРЫС С РАЗНОЙ УСТОЙЧИВОСТЬЮ К СТРЕССУ**

Л. Н. Рогова, В. Н. Поветкина

Волгоградский государственный медицинский университет, Российская Федерация

Acetate stomach ulcer was experimentally modeled in stress resistant and stress nonresistant rats for immunohistochemical identifying a specific number and expression intensity of eNOS-positive cells and determination of the magnesium level in biological media. The content of intra-erythrocyte magnesium in reaction with titanium yellow was reduced in rats with different resistance to stress in low eNOS activity in the gastric mucosa, in the muscular layer in stress nonresistant animals and almost complete absence of antigen-positive cells in the submucosa. A positive correlation was found between the level of intra-erythrocyte magnesium and the specific number of eNOS-positive cells in the gastric mucosa in both groups of animals and also between expression intensity in the submucosa of stress nonresistant rats. A relationship was established between the magnesium content, specific number and expression intensity of eNOS in stress nonresistant rats in the stomach submucosa.

**Keywords:** endothelial nitric oxide synthase, magnesium, acetate ulcer, stress resistant, stress nonresistant rats