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METHIDICAL APPROACHES TO BIOASSAY OF PHENOLIC HYDROXYLENES CONTAIN SUBSTANCES

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МЕТОДИЧЕСКИЙ ПОДХОД К АНАЛИЗУ ВЕЩЕСТВ, СОДЕРЖАЩИХ В СТРУКТУРЕ ФЕНОЛЬНЫЕ ГИДРОКСИЛЫ, ПРИ БИОАНАЛИТИЧЕСКИХ ИССЛЕДОВАНИЯХ

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This article describes method of development approaches for bioassay of substances containing stable and unstable phenolic hydroxyls using methyl dopa and mycophenolic acid for example

Keywords: phenolic hydroxyls, bioassay, mycophenolic acid, methyl dopa, stabilization

Описаны подходы к разработке биоаналитических методик для определения веществ, содержащих стабильные и нестабильные фенольные гидроксилы, на примере метилдопы и микофеноловой кислоты.

Ключевые слова: фенольные гидроксилы, биоаналитика, микофеноловая кислота, метилдopa, стабилизация

The main step of bioequivalence and pharmacokinetic 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 methyl dopa (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.

Fig. 1. Structures of mycophenolic acid (A) and methyl dopa (B)

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 the structure of methyl dopa. Addition of 10 % ascorbic acid solution is necessary to prevent degradation of catechins (polyphenolic compounds) and 3,4-dihydroxycinnamic acid (diatomic phenolic compound) in urine samples.

The aim of study was to develop of the methodological and approaches to bioassay of substances, containing phenolic hydroxyls in the structure.

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 x 3.0 mm, 5 µm) and Phenomenex Synergi Fusion RP 80Å (150x3.0 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 Agilent 1260 Infinity HPLC-MS system for quantification of mycophenolic acid consisted of G1311C pump, the G1329B ALS autosampler, the G1316A column thermostat and the 6130 Single quadrupole mass spectrometer equipped with JetStream electrospray ion source.

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.

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 this 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.

Table 1
Study of Back conversion of phenolic glucuronide of mycophenolic acid

	Mean peak area of LLOQ sample	K ₃ EDTA							
		Initial	1 h	2 h	3 h	4 h	6 h	8 h	24 h
Peak area of MPA	8073.5	0	339.6	485.3	520.4	1077.8	1374.7	1801.0	3632.7
% of the peak area of LLOQ sample	-	0	4.2	6.0	6.4	13.3	17.0	22.3	45.2
		Heparin							
		Initial	1 h	2 h	3 h	4 h	6 h	8 h	24 h
Peak area of MPA	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	-	8.0	18.5	23.9	54.8	75.2	120.6	154.3	8.0

Table 2

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 % 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 % 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, thiosulphate and metabisulphite in concentrations of 5 and 10 %, as well as a mixture of ascorbic acid, sodium sulfite, sodium hydroxycarbonate 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/internal standard» obtained before and after investigation.

Methyldopa was stable for 24 h at room temperature, and also during 3 freezethaw cycles after usage of ascorbic acid solutions in a concentration of 5 and 10 % and a solution containing a mixture of ascorbic acid, sodium sulfite sodium hydrocarbonate in concentrations of 5 %, 0.2 % and 2.4 %, respectively, in combination with K₃EDTA. 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.

Deproteinizates 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 deproteinizates 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 № 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).

Table 3

The results of validation tests of the developed methods

Method		Methyldopa (HPLC-MS/MS)	Mycophenolic acid (HPLC-MS)
Parameter		Results	
Selectivity		There was analysis of 6 samples of blank blood plasma obtained from different sources and plasma samples containing analytes at the LLOQ concentration level	
		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: 0.02 – 3.00 µg/ml. The correlation coefficient of the 8-point calibration curve (<i>r</i>) 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 (<i>r</i>) was ranged from 0.9982 to 0.9985
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 %	MF*** was ranged from 0.789 to 0.808; CV was ranged 2.96 to 5.60 %
Stability	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).

Fig. 4. Approaches to development of bioanalytical method for quantify of drugs containing phenolic hydroxylenes

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 pharmaco-

kinetic study of sodium mycophenolate in rats, and also was verified using the previously published HPLC-MS/MS method.

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INTERRELATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE ACTIVITY IN TISSUES OF THE STOMACH AND MAGNESIUM BALANCE IN THE PERIOD OF EROSION-ULCERATIVE ACID-INDUCED LESION DEVELOPMENT IN RATS WITH DIFFERENT RESISTANCE TO STRESS

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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

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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