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Research Article

Methodical approaches to bioassay of substances containing unstable functional groups

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Abstract

Introduction: This article describes the method development approaches for bioassay of substances containing unstable functional groups and forming unstable metabolites using the example of mycophenolic acid, methyldopa and mebeverine metabolites.

Materials and Methods: The concentration of mycophenolic acid, which contains one phenolic hydroxyl and forms glucuronides during metabolism, was measured in plasma using HPLC-MS/MS, HPLC-MS and GC-MS. The determination of methyldopa, containing two phenolic hydroxyls, in stabilised plasma was performed by HPLC-MS/MS in the range of 0.02-3.00 μg/ml. Desmethyl mebeverine acid, which contains one phenolic hydroxyl and is metabolised by forming phenolic glucuronide, was assayed simultaneously with mebeverine acid in the range of 10-2000 ng/ml.

Results and Discussion: The selection of storage conditions of the samples containing unstable substances should begin with selecting an anticoagulant based on the study of its short-term stability and freeze/thaw stability. If an unacceptable result is obtained, a combination of the anticoagulant and a stabiliser solution, as well as a concentration of this solution and its volume ratio to the biological fluid should be titrated. After which, this method should be validated by using the selected anticoagulant or the combination of the anticoagulant and stabiliser solution.

Conclusion: The application of this approach to developing a bioanalytical method for determination of unstable compounds makes it possible to avoid obtaining false assay results.

Keywords

bioanalytical studies, instability, mycophenolic acid, methyldopa, desmethyl mebeverine acid

Introduction

Stability of the analytes in biological matrices is an important component, which guarantees obtaining reliable results of bioanalytical studies. Thus, a wrong conclusion about the bioequivalence (BE) of the generic and original drug can lead to the registration of the drug which does not meet the requirements of effectiveness and safety. Errors of therapeutic drug monitoring may result in a faulty dosage (Khokhlov 2017), which puts patients' health at high risk.

Oxidation and hydrolysis are the main causes for the decomposition of molecules of drugs and their metabolites in biological fluids (Khokhlov 2017, Yaichkov et al.

2017, Dell 2004, Khokhlov et al. 2017a, Hilhorst et al. 2017, Li et al. 2013, Li et al. 2011). Stabilisation of biological samples may be achieved by changing the storage conditions, selecting an anticoagulant and adding stabilisers depending on the structure of the analyte: antioxidants, buffer solutions, acid solutions and bases for pH correction, enzyme inhibitors (Yaichkov et al. 2017, Dell 2004, Khokhlov et al. 2017a, Hilhorst et al. 2017, Li et al. 2013, Li et al. 2011). Substances which contain phenolic hydroxyls are the main examples of readily oxidisable compounds (Khokhlov et al. 2017a, Hilhorst et al. 2017). Back conversion of glucurone conjugates into the parent drugs is the most common example of the hydrolysis of compounds in biological fluids (Hilhorst et al. 2017, Li et al. 2011). Thus, over the last 5 years, results have been published for over 30 bioanalytical studies of drugs which metabolise by forming glucuronides (Yaichkov et al. 2017, Li et al. 2011, Brκtas et al. 2016, Veeragoni et al. 2016, Shah et al. 2016, Sengupta et al. 2017, Patel et al. 2017). The least stable are O-acylglucuronides (Yaichkov et al. 2017, Dell 2004, Hilhorst et al. 2017, Li et al. 2013, Li et al. 2011); however, phenolic glucuronides may also undergo hydrolytic decomposition (Li et al. 2011, Ye et al. 2017).

Therefore, mycophenolic acid (MPA) and desmethyl mebeverine acid (DMA) which both contain one phenolic hydroxyl and methyldopa (MD) which contains two phenolic hydroxyls (Fig. 1) were selected to develop approaches to bioassay of substances containing unstable functional groups.

Besides, MPA and DMA are conjugated to glucuronides during metabolism (Khokhlov 2017, Kristinsson et al. 1994, Khokhlov et al. 2016), which results in MPA forming acylglucuronide (AcMPAG) and phenolic glucuronide (MPAG) with DMA forming only phenolic glucuronide (DMAG). A literature review showed that there was no need to add antioxidant solutions to the samples of biological fluids (Khokhlov et al. 2016, Benoit-Biancamano et al. 2007, Brandhorst et al. 2016, Heinig et al. 2010, Oliveira et al. 2002, Ryna et al. 2001, Vlase et al. 2013, Valizadeh et al. 2010, Elliott and Burgess 2006). The results of studying back-conversion of MPAG and AcMPAG during the storage process differ: some authors emphasise the necessity to use buffer solutions to slow hydrolysis of these metabolites (Benoit-Biancamano et al. 2007, Brandhorst et al. 2016), whereas others state that degradation of MPA conjugates was not significant and adding stabilisers was not necessary (Khokhlov et al. 2016, Heinig et al. 2010). Therefore, studying this process is of current concern.

Materials and Methods

Sample preparation for HPLC-MS/MS-determination of MPA was performed using deproteinisation: 450 µl of the deuterated internal standard (IS) methanol solution of mycophenolic acid-D3 was added to 50 µl of plasma. The mixture was vortexed and centrifuged at 2500 rpm for 10 min. A Kinetex C18 column (30 * 4.6 mm, 2.6 µm) with

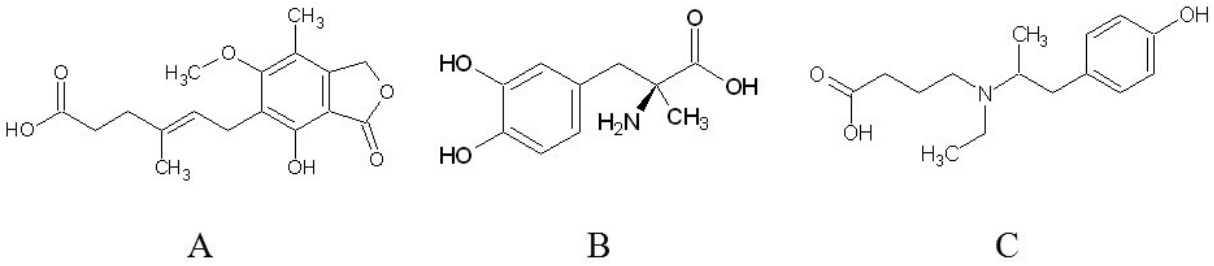

Figure 1. Graphic formulae of mycophenolic acid (**A**), methyldopa (**B**) and desmethyl mebeverine acid (**C**).

a Phenomenex Security guard (C18, 4 * 3 mm) and a mobile phase based on acetonitrile and water in the gradient elution mode were applied for the analysis. Mass spectrometry detection was carried out in negative ion MRM mode by the following MRM-transitions: MPA – 319 → 191+205 m/z; MPA-D_3 – 322 → 191 + 205 m/z (Khokhlov et al. 2016).

Protein precipitation was also used for preparing the samples for HPLC-MS-determination of MPA: 50 µl of plasma was vortexed with 200 µl of methanol. The mixture was centrifuged at 10000 rpm for 5 min. Separation was conducted on a Zorbax Eclipse Plus C18 column (100 * 4.6 mm, 3.5 µm) using a mobile phase based on acetonitrile, water and a 0.1% solution of formic acid (50:45:5, v/v/v) in the isocratic elution mode. Negative ions of MPA were detected in the SIM-mode by molecular ion – 319 m/z (Khokhlov et al. 2017a).

Liquid-liquid extraction by methylene chloride after correcting the pH value to 2.0 by phosphate buffer solution was used for preparing plasma samples for GC-MS-determination of MPA. After separation and evaporation of the extract, derivatisation of the analyte by a mixture of N,O-bis (trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane was performed. Chromatographic separation was done using Mega 5-MS column (25 m * 0.20 mm, 0.33 µm) under the following temperature programme: the initial temperature was 100°C for 3 min, heating was done at a rate of 25°C/min up to 300єC, the final temperature

was 300єC for 8 min (the total run time - 19 min). Mass spectrometric detection of di-TMS-derivative of MPA was performed in the SIM-mode – 449 m/z.

The method of HPLC-MS/MS was used to measure the concentration of DMA and a minor mebeverine metabolite – mebeverine acid (MA) in plasma. The samples were prepared by protein precipitation. A methanol solution (400 µl) of deuterated standards of mebeverine acid-D_5 (MA-D_5) and desmethyl mebeverine acid-D_5 (DMA-D_5) was added to an aliquot of plasma of 100 µl. The mixture was centrifuged at 3500 rpm for 10 min after vortexing. Chromatographic separation was performed on two columns Luna C8 Mercury (20*4.0 mm, 5 µm) and Luna 5u C8 (150*4.6 mm, 5 µm) using a mobile phase based on acetonitrile, methanol and formate buffer solution in the gradient elution mode. Mass spectrometry detection was carried out in positive ion registration mode by the following MRM-transitions: MA – 280→121 m/z, DMA – 266→107 m/z, MA-D_5 – 285→121 m/z and DMA-D_5 – 271→107 m/z (Khokhlov et al. 2017a).

The samples containing methyldopa for HPLC-MS/MS determination of its concentration were prepared by protein precipitation. First, 400 µl of deuterated internal standard of methyldopa-D_3 (MD-D_3) in methanol was added to 100 µl of plasma; then the mixture was vortexed and centrifuged at 3500 rpm and temperature of +4°C for 10 min. Chromatographic separation of the sample components was done using two columns Luna Phenyl-Hexyl (50*3.0 mm, 5 µm) and Synergi Fusion RP 80E (150*3.0 mm, 4 µm) in the isocratic elution mode. Mass spectrometry detection was carried out in positive ion registration mode by the following MRM-transitions: for MD – 212→139 m/z; for MD-D_3 (IS) 215→ 169 m/z (Khokhlov et al. 2017c).

Results and Discussion

At the beginning of developing the methods for determining MPA using HPLC-MS and HPLC-MS/MS, back-conversion of MPAG in the ion source was investigated. The selected chromatographic parameters for both methods allow separating the analyte and its main metabolite (Figs. 2 and 3); that is the reason why its fragmentation in the ionisation process does not affect the accuracy of measuring MPA. This experiment was not carried out when using the GC-MS method, because MPAG is not recovered from the plasma under the selected extraction conditions.

A preliminary evaluation of the stability of mycophenolic acid was made by applying the HPLC-MS/MS method in plasma samples at a concentration of 25.0 µg/ml using K_3EDTA and lithium heparinate as anticoagulants. The results obtained after 24 h of keeping the model mixtures at room temperature and 3 freeze/thaw cycles meet the acceptance criteria: mean concentration values of MPA at K_3EDTA were 99.8% and 100.6% of the initial concentration, respectively and at lithium heparinate-plasma they were 101.1% and 97.2% of the initial concentration, respectively. Thus, mycophenolic acid containing one phenolic hydroxyl is resistant to oxidation in blood plasma.

Back-conversion of MPAG during the storage process was also studied using these anticoagulants on the samples at a concentration of this metabolite of 100 µg/ml (Khokhlov et al. 2016). If the lower limit of quantification (LLOQ) of the method is 0.05 µg/ml, MPAG hydrolysis exceeds the maximum permissible level (20% of the MPA chromatographic peak area of the LLOQ sample) after 6 hours of storage at room temperature using K_3EDTA, which is significantly higher than with using lithium heparinate (Table 1). If LLOQ of the method is 0.5 µg/ml, back-conversion of MPAG is at the acceptable level when applying both anticoagulants. The LLOQ level of 0.5 µg/ml is sufficient for conducting of BE studies of mycophenolic acid formulations (Khokhlov et al. 2016). However, when using lithium heparinate, the hydrolysis degree of the metabolite was approximately three times higher. That is the reason why K_3EDTA was selected for further studies (Table 1).

The absence of influence of back-conversion of AcMPAG and MPAG on the accuracy of mycophenolic acid determination was also proved by re-analysis of the plasma samples obtained from rats for cross-validation. The difference between the initial and final results of measurements ranged from -3.43% to 9.49%, which does not exceed the maximum acceptable value of 20% (European Medicines Agency 2010, Mironov 2014, Council of the Eurasian Economic Commission 2016).

When developing a method for determining mebeverine metabolites in plasma, fragmentation of DMAG in the ionisation process was also studied. There was no decomposition of this metabolite in the ion source (Fig. 4).

The results of testing the stability of desmethyl mebeverine acid, containing one phenolic hydroxyl, in plasma after 24 hours by maintaining samples at room temperature and 3 freeze/thaw cycles, met the acceptance criteria. Back-conversion of DMAG in the samples with a concentration of 2000 ng/ml kept for 24 hours at room tem-

Table 1. Study of Back-Conversion of Phenolic Glucuronide of Mycophenolic Acid During Storage.

		% of the chrom. peak area of LLOQ sample			
		LLOQ of the method 0,5 µg/ml		LLOQ of the method 0,05 µg/ml	
		K_3EDTA	Lithium heparinate	K_3EDTA	Lithium heparinate
Storage time, h (room temperature)	2 h	-	-	6.0	23.9
	6 h	-	-	17.0	75.2
	24 h	2.58	7.44	45.2	154.3

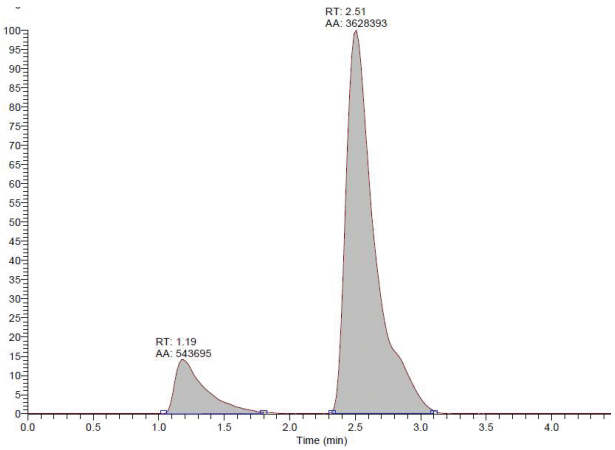

Figure 2. MRM-chromatogram of the mixture of MPA and MPAG when using HPLC-MS/MS method.

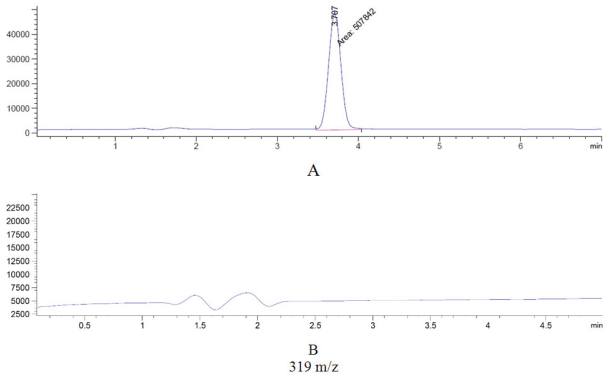

Figure 3. Chromatograms of MPA (**A**) and MPAG (**B**) solutions when using HPLC-MS method.

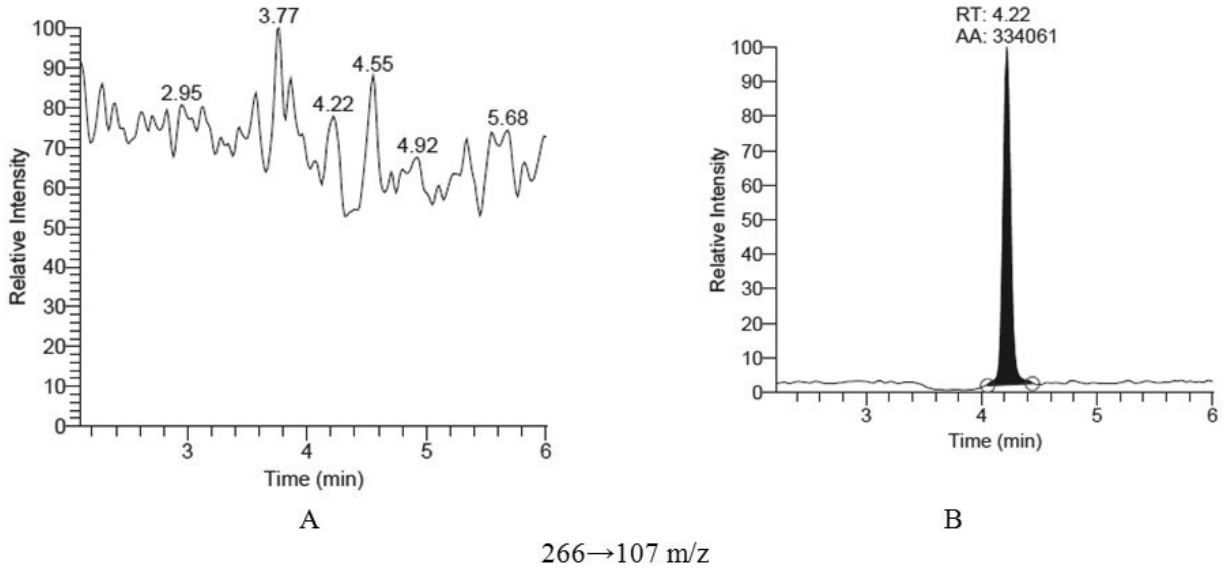

Figure 4. Chromatograms of DMAG (A) and DMA (B) solutions.

perature was completely absent. Consequently, addition of the stabiliser solution to the K_3EDTA plasma in this case is not required.

A preliminary study of the short-term stability and freeze/thaw stability of methyldopa, containing two phenolic hydroxyls, was carried out on plasma model mixtures at a concentration of 2.40 μg/ml. K_3EDTA and lithium heparinate were used as anticoagulants. The obtained results did not meet the acceptance criteria (Table 2). Therefore, it was necessary to add an antioxidant solution to the plasma to prevent oxidation of the analyte. The usage of solutions of ascorbic acid at a concentration of 5 and 10% and a solution containing a mixture of ascorbic acid, sodium sulphite and sodium bicarbonate at concentrations of 5%, 0.2% and 2.4%, respectively, used with K_3EDTA in the ratio of 1:5 (antioxidant solution/plasma), made it possible to prevent degradation of the substance (Table 2). The mixture of ascorbic acid, sodium sulphite and sodium bicarbonate was selected for further studies, because the sensitivity of the method was highest when using this mixture (Khokhlov et al. 2017a, Khokhlov et al. 2017c).

Thus, addition of an antioxidant stabiliser was necessary only when studying methyldopa, which contains two phenolic hydroxyls. The DMA molecule remained stable in plasma, despite the absence in the benzene ring of electron-withdrawing groups, which reduce the electron density and, thus, oxidation capacity. Back-conversion of the phenolic glucuronide of DMA was also completely absent. Hydrolysis of MPA conjugates, when using K_3EDTA as an anticoagulant, was at an acceptable level, which made it possible to avoid using buffer solutions to correct the pH of the medium.

Validation of the developed methods

The developed methods were validated in accordance with the requirements of EMA Guidelines (European Medicines Agency 2010), The Guidelines on the Eva-

Table 2. Selection of Stabilisers to Prevent Oxidation of Methyldopa.

Stabiliser	Concentration of stabiliser, %	Short-term stability (24 h at room temperature), n=2	Freeze/ thaw stability n=2	Short-term stability (24 h at room temperature), n=2	Freeze/ thaw stability n=2
		% of initial concentration			
		K_3EDTA		Lithium heparinate	
Without stabiliser	-	30.29	80.04	47.16	93.18
Ascorbic acid	5	88.44	102.30	80.91	99.69
	10	94.91	96.17	78.76	93.21
Mixture of ascorbic acid, sodium bicarbonate, sodium sulphite	5% ascorbic acid, 0.2% sodium sulphite, 2.4% sodium bicarbonate	89.31	98.24	72.48	95.07
Sodium thiosulphate	5	45.65	101.86	79.41	93.91
	10	31.32	99.81	83.46	97.78
Sodium metabisulphite	5	66.84	102.68	75.79	90.70
	10	75.99	97.25	73.22	88.70

luation of Medicinal Products by The Scientific Centre for Expert Evaluation of Medicinal Products of the Ministry of Healthcare of the Russian Federation (Vol. 1) (Mironov 2014) and Decision of the Council of the Eurasian Economic Commission № 85 "On Approval of the Rules for Conducting Bioequivalence Studies on Medicines in the Eurasian Economic Union" (Council of the Eurasian Economic Commission 2016). The results of validation tests meet all the acceptance criteria (Table 3).

Thus, the selected anticoagulants (for MPA and DMA) and the combination of an anticoagulant with a stabiliser solution (for MD) provide stability of the analytes in plasma samples for 24 hours at room temperature, 3 freeze/ thaw cycles and under freezing conditions (Table 4). The usage of a storage temperature not higher than -80°C made it possible to increase the storage period of samples containing methyldopa by up to 3 months.

Application of the developed methods

The HPLC-MS/MS method of MPA determination was applied for conducting a bioequivalence study of coated tablets of mycophenolate sodium (Khokhlov et al. 2016). The HPLC-MS and GC-MS-methods of quantification of mycophenolic acid were tested by cross validation in rat plasma samples. The method developed for measuring MD concentrations in plasma was used in an open randomised cross-over study of bioequivalence of methyldopa tablet formulations (Khokhlov et al. 2017a). The method of determining MA and DMA in plasma was applied to conduct a pharmacokinetics study of mebeverine sustained release capsules (Khokhlov et al. 2017b).

The statistical analysis was performed using StatSoft STATISTICA v.10, R package, Bear module (Lee, Hsinya and Lee, Yung-jin, bear: Data Analysis Tool for Average Bioequivalence and Bioavailability) and Microsoft Excel 2007.

Results of cross validation of methods for determining mycophenolic acid

The study was carried out on 10 white mongrel male rats weighing 250±10 g in accordance with the principles of Good Laboratory Practice (Ministry of Healthcare of the Russian Federation 2016). The aqueous solution of mycophenolate sodium was administered to rats orally in the dosage of 33.0 mg/kg. Blood sampling was performed 2 h after administration, as it is the time of MPAG and AcMPAG peak concentrations in plasma (Liu et al. 2017). Part of the plasma obtained after centrifusion was subjected to immediate sample preparation. The HPLC-MS/MS-method was chosen as a reference method, because to calculate the concentrations of MPA, a stable isotope-labelled internal standard MPA-D_3 was used which is the most preferable in accordance with the Eurasian Economic Union Guidelines for conducting BE studies (Council of the Eurasian Economic Commission 2016).

The relative concentrations of MPA obtained by the HPLC-MS-method were within the range of 95.18-106.93% compared to the HPLC-MS/MS results; concentrations obtained by the GC-MS-method were within the range of 94.27-110.26% (Table 5). It meets the acceptance criteria (European Medicines Agency 2010, Mironov 2014, Council of the Eurasian Economic Commission 2016): relative error does not exceed ± 20% for over 67% of samples. Thus, HPLC-MS and GC-MS methods developed for MPA determination provide accurate results without using a stable isotope-labelled internal standard for calculating the concentration.

Results of bioequivalence study of methyldopa tablet formulations

The study of comparative pharmacokinetics of methyldopa formulations was conducted on 24 healthy volunteers. The test drug was Methyldopa tablets (250 mg) (R-Pharm,

Table 3. Validation Results of Methods Developed.

Parameter		Mycophenolic acid			Mebeverine metabolites (HPLC-MS/MS)		Methyldopa (HPLC-MS/MS)
		HPLC-MS/MS	HPLC-MS	GC-MS	Mebeverine acid	Desmetyl mebeverine acid	
Selectivity		Interference in the retention times of analytes did not exceed 20% of the LLOQ level, in the retention times of internal standards (except for HPLC-MS and GC-MS methods of determining MPA) did not exceed 5% of the average peak area.					
LLOQ		0.5 μg/ml	0.05 μg/ml	0.05 μg/ml	10 ng/ml	10 ng/ml	0.02 μg/ml
Calibration curve		0.5-30.0 μg/ml	0.05-30.00 μg/ml	0.05-30.00 μg/ml	10-2000 ng/ml	10-2000 ng/ml	0.02-3.00 μg/ml
Accuracy (relative error)		-4.03 ч +12.85%.	-14.02% ч +11.82%	-14.35% ч +6.82%	-11.83% ч +13.86%	-1.73% ч +13.77%	-5.28% ч +7.42%
Precision (CV, %)		1.72% ч 8.52%	0.36% ч 9.82%	1.95% ч 5.58%	1.40% ч 8.24%	1.40%ч 8.58%	0.69% ч 3.97%
Matrix effects (CV NMF)	LQC	7.46%	2.96% (MF)	2.28% (MF)	3.66%	1.59%	0.38%
	HQC	6.55%	5.60% (MF)	2.80% (MF)	5.47%	2.82%	1.45%
Dilution integrity (relative error)		-6.47% ч +5.71%	-9.46% ч +4.22%	-0.84% ч +10.99%	-2.04% ч +1.88%	+0.64% ч +4.13%	-1.94% ч +3.27%

Note: LLOQ - lower limit of quantification, LQC- low QC samples; HQC - high QC samples; NMF – normalised matrix factor; MF- matrix factor

Table 4. Stability Study of Analytes in Blood Plasma.

Parameter			Mycophenolic acid		Mebeverine metabolites				Methyldopa	
					MA		DMA			
Short-term stability (24 h), % initial conc.		LQC	99.18		107.72		106.33		95.50	
		HQC	99.46		97.81		98.54		89.58	
Freeze/ thaw stability (3 cycles), % initial conc.		LQC	105.10		111.83		105.44		104.43	
		HQC	103.55		102.45		102.79		106.95	
Autosampler stability, % initial conc.		LQC	107.72		109.06		103.44		101.91	
		HQC	108.07		97.44		94.96		101.28	
Long-term stability, % initial conc.	not exceeding -20˚C	LQC	100.38 (1 month)	103.24 (4 months)	111.56 (1 month)	109.33 (4 months)	108.61 (1 month)	105.83 (4 months)	90.74 (1 month)	66.92 (3 months)
		HQC	94.79 (1 month)	100.86 (4 months)	105.45 (1 month)	103.56 (4 months)	110.33 (1 month)	110.06 (4 months)	86.20 (1 month)	59.57 (3 months)
	not exceeding -80˚C	LQC	-	-	-	-	-	-	100.60 (1 month)	100.76 (3 months)
		HQC	-	-	-	-	-	-	99.12 (1 month)	94.08 (3 months)

Russia, batch number 20614, expiry date: 07.2016) and a reference preparation was Dopegit (Egis, Hungary, batch number D358N1014, expiry date: 10.2019). Blood samples was taken pre-dose and post-dose at 5 min, 15 min, 30 min, 45 min, 1 h, 1 h 15 min, 1 h 30 min, 1 h 45 min, 2 h, 2 h 15 min, 2 h 30 min, 3 h, 4 h, 6 h, 9 h, 12 h, 18 h and 24 h after drug administration in vacuum tubes with K_3EDTA. Then the blood samples were immediately centrifuged at a speed of 3000 rpm and a temperature of +4°C for 5 minutes. A stabiliser solution was added to the obtained plasma. A total of 864 samples were analysed using the developed method.

The values of the pharmacokinetic parameters of the drugs under study are not statistically significantly different (Table 6) and their average pharmacokinetic profiles practically coincide (Fig. 5). The calculated values of the 90%-confidence intervals of the parameters C_{max}, AUC_{0-t}, C_{max}/AUC_{0-t} are within the acceptable limits of 80.0-125.0% (Table 7). The coefficient of intra-subject variability of C_{max} was 33.10%, which conformed to the level of a highly variable drug. The results of multivariate analysis of variance (ANOVA) show that the main factor contributing to the variability of this parameter was the subjects themselves ($p = 0.027$). Incurred samples re-analysis (of 96 samples - 11.1% of the total amount) showed 100% reproducibility of the analytical method.

Thus, Methyldopa (by R-Pharm, Russia) and Dopegit (by Egis, Hungary) are bioequivalent. The measures developed for stabilisation of the analyte ensure validity of the results obtained.

Results of the pharmacokinetic study of mebeverine sustained release capsules

The cohort for assessing pharmacokinetic parameters of MA and DMA after administration of sustained release capsules of Duspatalin (200 mg) (by Abbott Healthcare SAS, France, batch number 10215, expiry date: 04.17) included 24 subjects aged 18-45 years. Blood samples were taken pre-dose and post-dose at 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 5 h, 6 h, 8 h, 10 h, 12 h and 24 h after drug administration in vacuum tubes with K_3EDTA. Then the plasma was immediately separated by centrifusion and frozen. A total of 360 samples were analysed.

Thus, the obtained data about the pharmacokinetic parameters of mebeverine metabolites after taking sustained release capsules can be used to develop a bioequivalence study design of studies (Table 8, Fig. 6)

Table 5. Cross Validation of Methods for Determining Mycophenolic Acid in Plasma.

	Determinations by HPLC-MS/MS method	Determinations by HPLC-MS method		Determinations by GC-MS-method	
№ of sample		**Abs. µg/ml**	**Rel. %**	**Abs. µg/ml**	**Rel. %**
1	9.82	9.35	95.18	9.63	98.11
2	8.65	8.96	103.55	8.16	94.27
3	5.64	5.18	91.86	5.57	98.80
4	9.61	9.66	100.58	10.38	108.01
5	12.36	12.31	99.62	11.86	95.94
6	9.02	9.01	99.81	9.57	105.99
7	4.63	4.95	106.93	4.62	99.82
8	20.09	21.40	106.54	19.60	97.54
9	9.18	9.65	105.05	10.01	109.00
10	7.44	7.89	106.10	8.20	110.26
Mean			101.77	-	101.52

Table 6. Main Pharmacokinetic Parameters of Test and Reference Methyldopa Drug.

Pharmacokinetic parameters	**Test drug (Mean± SD)**	**Reference drug (Mean± SD)**	**Criteria of comparison**	**p**
C_{max}, µg/ml	1.227±0.601	1.233±0.419	Student's t-test for dependent	0.952
AUC_{0-t}, µg*h/ml	6.219±3.080	6.385±2.153	group	0.740
$AUC_{0-\infty}$, µg*h/ml	6.436±3.181	6.529±2.187		0.997
C_{max}/AUC_{0-t}, h^{-1}	0.202±0.038	0.198 ±0.037		0.663
K_{el}, h^{-1}	0.229 ±0.110	0.207±0.107		0.127
T_S, h	3.89±2.13	4.45±2.37		0.056
MRT, h	4.97±0.77	4.98±0.76		1.000
T_{max}, h	3.18±1.23	2.90±0.86	Wilcoxon signed-rank test	0.469

Note: C_{max} - maximum plasma concentration in blood; T_{max} - time-to-peak concentration; AUC_{0-t} - area under the pharmacokinetic concentration-time curve from time zero to the last blood sampling procedure; $AUC_{0-\infty}$ - area under the pharmacokinetic curve from time zero to infinity; C_{max}/AUC_{0-t} - relative absorption rate; K_{el} - terminal elimination rate constant; T_S - drug elimination half-life; MRT - mean residence time.

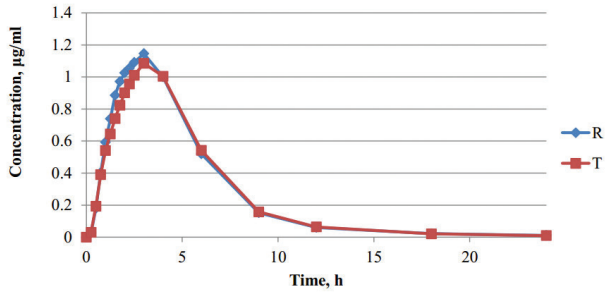

Figure 5. Averaged pharmacokinetic profiles of methyldopa concentrations in blood plasma after administration of test (T) and reference (R) drug.

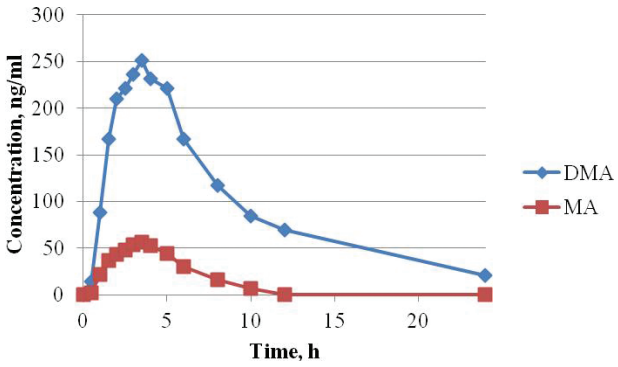

Figure 6. Averaged pharmacokinetic profiles of MA and DMA concentrations in blood plasma after administering a single dose of Duspatalin (200 mg).

Table 7. Results of Comparison of Bioequivalence of Methyldopa formulations.

Parameter	Geometric mean ratio	90% confidence intervals				Intra-subject variability coefficient
		Estimated value		Acceptable value		
		Lower limit	Upper limit	Lower limit	Upper limit	
AUC_{0-t}	92.93%	80.69%	107.03%	80.00	125.00	29.08%
C_{max}	94.89%	80.88%	111.34%	80.00	125.00	33.10%
C_{max}/AUC_{0-t}	102.11%	93.95%	110.98%	80.00	125.00	16.92%

Table 8. Pharmacokinetic Parameters of Mebeverine Metabolites.

	Mebeverine acid								
	C_{max}, ng/ml	T_{max}, h	AUC_{0-t}, ng·h/ml	$AUC_{0-\infty}$, ng·h/ml	$AUC_{0-t}/AUC_{0-\infty}$, %	C_{max}/AUC_{0-t}, h^{-1}	K_{el}, h^{-1}	$T_{1/2}$, h	MRT, h
Mean±SD	62.52±	3.27±	293.94±	365.85±	82.34±	0.2238±	0.27584±	2.87±	5.82±
	35.01	1.03	151.78	140.49	11.61	0.0598	0.08426	1.39	1.57
Min	22.3	1.5	54.03	110.01	47.43	0.1649	0.08683	1.61	4.27
Max	173.5	5	601.03	649.49	96.04	0.4128	0.43138	7.98	11.45
CV %	56.00	31.55	51.64	38.40	14.10	26.72	30.55	48.38	26.91
	Desmethyl mebeverine acid								
	C_{max}, ng/ml	T_{max}, h	AUC_{0-t}, ng·h/ml	$AUC_{0-\infty}$, ng·h/ml	$AUC_{0-t}/AUC_{0-\infty}$, %	C_{max}/AUC_{0-t}, h^{-1}	K_{el}, h^{-1}	$T_{1/2}$, h	MRT, h
Mean±SD	291.81±	3.19±	2191.85±	2551.74±	86.26±	0.1339±	0.11605±	7.52±	11.2±
	125.92	1.48	542.94	546.96	11.62	0.0497	0.06657	3.31	4.25
Min	114.6	1.5	1081.45	1388.65	40.34	0.0838	0.04631	2.33	5.34
Max	607.4	8	3262.43	3547.35	95.84	0.2969	0.29771	14.97	22.49
CV %	43.15	46.44	24.77	21.43	13.47	37.15	57.36	44.04	37.97

Conclusions

The method for the quantitative determination of substances forming such metabolites as glucuronides, N-oxides, esters, lactones and also for the simultaneous analysis of esters and lactones with their acid forms should ensure that there is no influence of degradation of these compounds in the ion source on the results of measuring the analyte concentration. The selection of storage conditions for samples containing substances with unstable functional groups should begin by selecting an anticoagulant, based on a study of short-term stability and freeze/thaw stability of the analyte and back-conversion of its metabolites. When obtaining an unacceptable result, it is necessary to select a combination of an anticoagulant and a stabiliser solution, as well as a concentration of this solution and its volume ratio to the biological fluid. After this, the developed method should be validated using the selected anticoagulant or a combination of the anticoagulant and stabiliser solution.

Thus, the application of this approach to developing a bioanalytical method for determination of compounds containing unstable functional groups makes it possible to avoid obtaining false assay results. This significantly reduces the risk to the health of patients when administering generic drugs or when using the results of therapeutic drug monitoring for correcting the dosage.

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