Methodical approaches to bioassay of substances containing unstable functional groups

Alexander L. Khokhlov¹, Ilya I. Yaichkov¹,², Yuriy A. Dzhurko³, Leonid N. Shitov¹,³, Anastasia A. Shitova³

¹ Yaroslavl State Medical University, 5 Revolyutsionnaya St., Yaroslavl 150000 Russia
² Yaroslavl State Pedagogical University named after K.D. Ushinsky, 108/1 Respublikanskaya St., Yaroslavl 150000 Russia
³ Quinta-Analytica Yaroslavl LLC, 52g Leningradskaya St., Yaroslavl 150045 Russia

Corresponding author: Ilya I. Yaichkov (ilya_1993_08@mail.ru)

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.)
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, Benoît-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 (Benoît-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 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 300°C, the final temperature was at a rate of 25°C/min up to 300°C, the final temperature was...
was 300°C 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-D₃ (MA-D₃) and desmethyl mebeverine acid-D₃ (DMA-D₃) 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-D₃ – 285→121 m/z and DMA-D₃ – 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-D₃ (MD-D₃) 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-D₃ (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 K₃EDTA 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 K₃EDTA 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 K₃EDTA, 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 K₃EDTA 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>
<thead>
<tr>
<th>Table 1. Study of Back-Conversion of Phenolic Glucuronide of Mycophenolic Acid During Storage.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>% of the chrom. peak area of LLOQ sample</strong></td>
</tr>
<tr>
<td><strong>LLOQ of the method 0.5 μg/ml</strong></td>
</tr>
<tr>
<td><em>K₃EDTA</em></td>
</tr>
<tr>
<td>Storage time, h</td>
</tr>
<tr>
<td>(room temperature)</td>
</tr>
<tr>
<td>LLOQ of the method 0.5 μg/ml</td>
</tr>
<tr>
<td>K₃EDTA</td>
</tr>
<tr>
<td>Lithium heparinate</td>
</tr>
<tr>
<td>LLOQ of the method 0.05 μg/ml</td>
</tr>
<tr>
<td>K₃EDTA</td>
</tr>
<tr>
<td>Lithium heparinate</td>
</tr>
</tbody>
</table>
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$_3$EDTA 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, 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$_3$EDTA 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-

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, Hsin-ya 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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mycophenolic acid</th>
<th>Mebeverine metabolites (HPLC-MS/MS)</th>
<th>Methyldopa (HPLC-MS/MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC-MS/MS</td>
<td>HPLC-MS</td>
<td>GC-MS</td>
</tr>
<tr>
<td></td>
<td>LLOQ</td>
<td>Calibration curve</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 μg/ml</td>
<td>0.05-30.0 μg/ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.05 μg/ml</td>
<td>0.05-30.0 μg/ml</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td></td>
<td>0.05 μg/ml</td>
<td>10-2000 μg/ml</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td></td>
<td>10 ng/ml</td>
<td>10-2000 μg/ml</td>
<td>0.02 μg/ml</td>
</tr>
<tr>
<td>Selectivity</td>
<td>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.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLOQ</td>
<td>0.5 μg/ml</td>
<td>0.05-30.0 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Calibration curve</td>
<td>0.05-30.0 μg/ml</td>
<td>0.05-30.0 μg/ml</td>
<td>10-2000 μg/ml</td>
</tr>
<tr>
<td>Accuracy (relative error)</td>
<td>-4.03 ±12.85%</td>
<td>-14.02% ±11.82%</td>
<td>-11.83% ±13.86%</td>
</tr>
<tr>
<td>Precision (CV, %)</td>
<td>1.72% ±8.52%</td>
<td>0.36% ±9.82%</td>
<td>1.40% ±8.24%</td>
</tr>
<tr>
<td>Matrix effects (CV NMF)</td>
<td>LQC</td>
<td>7.46%</td>
<td>2.96% (MF)</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>6.55%</td>
<td>5.60% (MF)</td>
</tr>
<tr>
<td>Dilution integrity (relative error)</td>
<td>-6.47% ±5.71%</td>
<td>-9.46% ±4.22%</td>
<td>-0.84% ±10.99%</td>
</tr>
<tr>
<td>Note: LLOQ - lower limit of quantification, LQC - low QC samples; HQC - high QC samples; NMF – normalised matrix factor; MF- matrix factor</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Stability Study of Analytes in Blood Plasma.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mycophenolic acid</th>
<th>Mebeverine metabolites</th>
<th>Methyldopa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LQC</td>
<td>MA</td>
<td>DMA</td>
</tr>
<tr>
<td>Short-term stability</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(24 h), % initial conc.</td>
<td>LQC</td>
<td>99.18</td>
<td>107.72</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>99.46</td>
<td>97.81</td>
</tr>
<tr>
<td>Freeze/ thaw stability</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3 cycles), % initial conc.</td>
<td>LQC</td>
<td>105.10</td>
<td>111.83</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>103.55</td>
<td>102.45</td>
</tr>
<tr>
<td>Autosampler stability, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% initial conc.</td>
<td>LQC</td>
<td>107.72</td>
<td>109.06</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>108.07</td>
<td>97.44</td>
</tr>
<tr>
<td>Long-term stability, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% initial conc.</td>
<td>not exceeding</td>
<td>LQC 100.38</td>
<td>103.24</td>
</tr>
<tr>
<td></td>
<td>-20˚C (1 month)</td>
<td></td>
<td>(4 months)</td>
</tr>
<tr>
<td></td>
<td>(4 months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC 94.79</td>
<td>100.86</td>
<td>105.45</td>
</tr>
<tr>
<td></td>
<td>(1 month)</td>
<td>(4 months)</td>
<td>(1 month)</td>
</tr>
<tr>
<td></td>
<td>not exceeding</td>
<td>LQC -</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-80˚C (1 month)</td>
<td></td>
<td>(3 months)</td>
</tr>
<tr>
<td></td>
<td>HQC -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5. Cross Validation of Methods for Determining Mycophenolic Acid in Plasma.

<table>
<thead>
<tr>
<th>№ of sample</th>
<th>Determinations by HPLC-MS/MS method</th>
<th>Determinations by HPLC-MS method</th>
<th>Determinations by GC-MS-method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Abs. μg/ml</td>
<td>Rel. %</td>
<td>Abs. μg/ml</td>
</tr>
<tr>
<td>1</td>
<td>9.82</td>
<td>95.18</td>
<td>9.63</td>
</tr>
<tr>
<td>2</td>
<td>8.65</td>
<td>103.55</td>
<td>8.16</td>
</tr>
<tr>
<td>3</td>
<td>5.64</td>
<td>91.86</td>
<td>5.57</td>
</tr>
<tr>
<td>4</td>
<td>9.61</td>
<td>100.58</td>
<td>10.38</td>
</tr>
<tr>
<td>5</td>
<td>12.36</td>
<td>99.62</td>
<td>11.86</td>
</tr>
<tr>
<td>6</td>
<td>9.02</td>
<td>99.81</td>
<td>9.57</td>
</tr>
<tr>
<td>7</td>
<td>4.63</td>
<td>99.62</td>
<td>9.57</td>
</tr>
<tr>
<td>8</td>
<td>20.09</td>
<td>106.54</td>
<td>19.60</td>
</tr>
<tr>
<td>9</td>
<td>9.18</td>
<td>105.05</td>
<td>10.01</td>
</tr>
<tr>
<td>10</td>
<td>7.44</td>
<td>106.10</td>
<td>8.20</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>101.77</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Test drug (Mean± SD)</th>
<th>Reference drug (Mean± SD)</th>
<th>Criteria of comparison</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{max}, μg/ml</td>
<td>1.227±0.601</td>
<td>1.233±0.419</td>
<td>Student’s t-test for dependent</td>
<td>0.952</td>
</tr>
<tr>
<td>AUC_{0-τ}, μg*h/ml</td>
<td>6.219±3.080</td>
<td>6.385±2.153</td>
<td>group</td>
<td>0.740</td>
</tr>
<tr>
<td>AUC_{0-∞}, μg*h/ml</td>
<td>6.436±3.181</td>
<td>6.529±2.187</td>
<td></td>
<td>0.997</td>
</tr>
<tr>
<td>C_{max}/AUC_{0-τ}, h^{-1}</td>
<td>0.202±0.038</td>
<td>0.198±0.037</td>
<td></td>
<td>0.663</td>
</tr>
<tr>
<td>K_e, h^{-1}</td>
<td>0.229±0.110</td>
<td>0.207±0.107</td>
<td></td>
<td>0.127</td>
</tr>
<tr>
<td>T_{1/2}, h</td>
<td>3.89±2.13</td>
<td>4.45±2.37</td>
<td></td>
<td>0.056</td>
</tr>
<tr>
<td>MRT, h</td>
<td>4.97±0.77</td>
<td>4.98±0.76</td>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td>T_{max}, h</td>
<td>3.18±1.23</td>
<td>2.90±0.86</td>
<td>Wilcoxon signed-rank test</td>
<td>0.469</td>
</tr>
</tbody>
</table>

Note: C_{max} - maximum plasma concentration in blood; T_{max} - time-to-peak concentration; AUC_{0-τ} - area under the pharmacokinetic concentration-time curve from time zero to the last blood sampling procedure; AUC_{0-∞} - area under the pharmacokinetic curve from time zero to infinity; C_{max}/AUC_{0-τ} - relative absorption rate; K_e - terminal elimination rate constant; T_{1/2} - drug elimination half-life; MRT - mean residence time.
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.
References


Author Contributions

Alexander L. Khokhlov, Doctor of Medical Sciences, Professor, Corresponding member of The Russian Academy of Sciences, Head of the Department of Clinical Pharmacology, e-mail: al460935@yandex.ru. The author set the goals and objectives of the clinical part of the bioequivalence and pharmacokinetics studies and developed the design of these studies.

Ilya I. Yaichkov, post-graduate student of the Department of Clinical Pharmacology, Junior Research Fellow of M.V. Dorogov Centre for Transfer of Pharmaceutical Technologies, Yaroslavl State Pedagogical University named after K.D. Ushinsky, e-mail: ilya_1993_08@mail.ru. The author set the goal and objectives of the bioanalytical part of the study, did a literature review, took part in developing and validating the methods, as well as in carrying out the bioassay using the methods.

Yuriy A. Dzhurko, PhD in Pharmacy, Senior Analyst, Quinta-Analytica Yaroslavl LLC, e-mail: y.dzhurko@qayar.ru. The author took part in developing and validating the methods, as well as in carrying out the bioassay using the methods.

Leonid N. Shitov, PhD in Biology, Head of Bioanalytical laboratory Quinta-Analytica Yaroslavl LLC, Assistant at the Department of Polyclinical Therapy and Clinical Laboratory Diagnostics, Yaroslavl State Medical University, e-mail: schitov@inbox.ru. The author took part in developing and validating the methods, as well as in carrying out the bioassay using the methods.

Anastasia M. Shitova, PhD in Physics and Mathematics, statistician Quinta-Analytica Yaroslavl LLC, e-mail: am_schitova@cphinvest.ru. The author did the statistical processing of the data obtained in the bioequivalence and pharmacokinetics studies.