Short communication

Optimization of the Extraction Procedure of Apixaban from Dried Rat Plasma Spots

ABSTRACT (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Aims: Apixaban is an anticoagulant used to treat and prevent blood clots, as well as to prevent stroke in people with atrial fibrillation. The dried spot analyses, including dried blood spots and dried plasma spots, are used to simplify techniques for determining drug concentrations in blood and plasma. In this case, equipment with highly sensitive detector is required, for example, mass spectrometer, as well as a high level of drug extraction from the dried spot. In this work, apixaban extraction from dried plasma spots (DPS) was studied in order to determine the optimal parameters of the extraction method.
Study design: Short Research Articles.
Place and Duration of Study: Core Facility of Mass Spectrometric Analysis, Institute of Chemical Biology and Fundamental Medicine SB RAS, between September 2019 and February 2020
Methodology: The organic extraction method was chosen for evaluation as the most suitable for LC-MS assay. Several parameters: percentage of organic solvent, presence or absence of 0.1% formic acid (FA), time, volume and temperature of extraction were investigated to find the best combination for recovery of apixaban from DPS for further LC-MS analysis.
Results: The results showed that the main influence on the extraction is the composition and volume of the solvent, temperature and time. Pure acetonitrile is the worst solvent for extracting apixaban from DPS. Solvents: MeOH:H_2O (100:0, v: v), MeOH: 0.1% FA in H_2O (80:20, v:v), ACN: 0.1% FA in H_2O (90:10, v:v) or ACN:MeOH (90:10, v:v) provide better recovery of apixaban. The most optimal solvent is 90% acetonitrile with an extraction temperature of 40 °C, an extraction time of 15 minutes, and a solvent volume of 100 µl.
Conclusion: Several solvents acceptable for LC-MS analysis with optimized recovery parameter from DPS can be used for routine extraction of apixaban.

Keywords: Dried plasma spot, DPS, DBS, apixaban, LC-MS/MS, extraction

1. INTRODUCTION

Atrial fibrillation is the most frequent disorder of heart rhythm associated with risk increasing of stroke and systemic thromboembolism by 5 times, and death by half [1]. For decades, vitamin K antagonists such as warfarin and phenprocoumon have served as oral anticoagulants to treat and prevent thromboembolic disorders [2]. Though anticoagulants are certainly effective in prevention thromboembolic complications in atrial fibrillation, the frequency of their application remains unacceptably low. The situation began to change radically starting in 2010, when the so-called new oral anticoagulants appeared on the market [3–5]. Their advantages are wide therapeutic index, fixed dose regimen, favorable efficacy/safety ratio, and minor drug−drug and drug−food interactions [6]. For example, apixaban is a selective inhibitor of blood clotting human factor-Xa [7, 8]. It is also used to treat deep veins and pulmonary embolism and to prevent their recurrence [9]. The chemical structure of apixaban is presented at Fig.1.

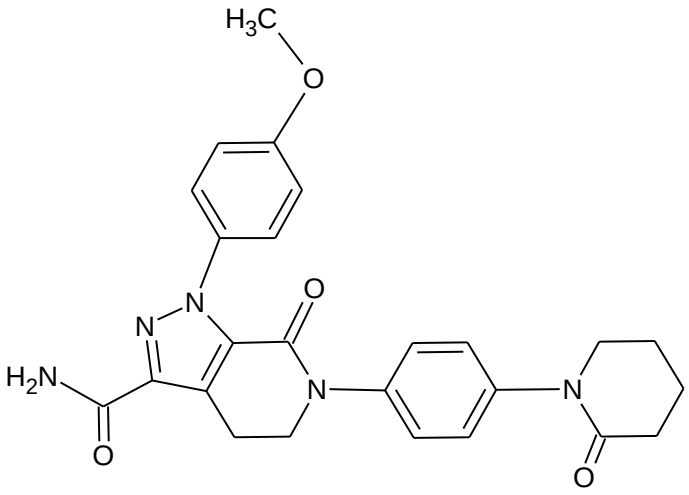


Figure 1. Chemical structure of apixaban.

Currently, dried blood spots (DBS) technic is widely used in screening analysis, as well as for routine clinical research [6, 10, 11]. This method has various advantages, such as minimal invasiveness, minimal risk of infection with infectious pathogens, and ease of storage and transportation. Therefore, this method together with mass spectrometry is convenient to use for preclinical or clinical pharmacokinetic studies [12–14]. However, the difference in the hematocrit values in human blood can negatively affect the measured concentration of drugs. Dried plasma spots (DPS) can be used to solve this problem. There is one work [6], where authors used the postcolumn infused internal standard with LC-MS/MS method to estimate the concentration of apixaban in DBS, but there are no studies of quantitative determination of apixaban in DPS. One of the first steps in determining the concentration of apixaban is its extraction from DPS. So the aim of this study was to find the optimized parameters of apixaban extraction from DPS.

2. MATERIAL AND METHODS

2.1. Reagents.

Apixaban, Whatman 903 Protein Saver Card and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol of LC-MS grade were purchased from Panreac AppliChem (Barcelona, Spain). Water was purified by means of a Milli-Q system from Millipore Corp. (Bedford, USA). Nitrogen gas (ultrapure, >99.9%) was produced by an Agilent 5183-2003 nitrogen generator (Agilent Technologies, USA).

2.2. Equipment and HPLC-MS/MS Conditions.

Mass spectrometry analysis was carried out in the Core Facility of Mass Spectrometric Analysis (ICBFM SB RAS). Chromatographic separation of the samples was achieved using an Agilent 1200 HPLC (Agilent Technologies, USA). Sample injection volume was 10 µl. The flow rate was 0.4 mL/min and the gradient was composed of water containing 0.1% (v:v) formic acid (eluent A) and methanol containing 0.1% (v:v) formic acid (eluent B). Analysis was carried out in isocratic elution mode with 50% B. The run time was 2 min. The autosampler temperature was held at 4°C.

MS/MS detection was performed on an Agilent 6410 QQQ mass spectrometer (Agilent Technologies, USA). Analytes were detected in positive ionization mode using multiple reaction monitoring. The capillary voltage was set to 4000 V, and the gas temperature was set to 300 °C. The nebulizer gas pressure and flow were 30 psi and 8 L/min, respectively. Dwell time was set to 200 ms. The ion transitions for apixaban were m/z 267.2→145.1 (collision energy 25 V, fragmentor voltage 135 V) as a quantifier; m/z 267.2→190.1 (collision energy 15 V, fragmentor voltage 135 V) and m/z 267.2→225 (collision energy 7 V, fragmentor voltage 135 V) as qualifiers. Signal output was captured and processed with the MassHunter software v.3.0. All LC-MS measurements were performed in duplicate.

2.3. Preparation of Samples.

Stock solution and working samples were prepared in same way as described in work [14]. Briefly, apixaban was dissolved in 70% acetonitrile to prepare a 10 mg/mL stock solution. The apixaban stock solution was diluted with 70% acetonitrile to prepare intermediate stock solution that was added to blank rat plasma to create working solution with apixaban concentration of 400 ng/mL. All stock and working solutions were freshly made on the day of the analysis and

were stored at 4 °C before use. The working samples with final plasma concentration of apixaban of 400 ng/mL (each consisting of 25 μL of rat plasma) was spotted on a Whatman 903 Protein Saver Card (GE Healthcare, USA) to fill the circles on the card and was air dried completely overnight. After that, 3.2 mm disks of DPS were cut out by means of a DBS Puncher, and each disk was placed in a 1.5 mL Eppendorf tube.

2.4. Solvents preparation.

Since there is no data on apixaban extraction, the five different types of solvents were chosen and prepared according to work [11]. The first one: MeOH:H_2O mixture from 50% to 100% of MeOH (v:v) with 10% step. The second one: MeOH: 0,1% of FA in H_2O mixture from 50% to 100% of MeOH (v:v) with 10% step. The third one: ACN:H_2O mixture from 50% to 100% of ACN (v:v) with 10% step. The fourth one: MeOH:0,1% of FA in H_2O mixture from 50% to 100% of ACN (v:v) with 10% step. The last one: MeOH:ACN mixture from 0% to 100% of MeOH (v:v) with 10% step.

2.5. Extraction procedure.

There was used the organic extraction method to optimize the extraction parameters. In general, organic solvent directly adds to DPS samples and then extraction is carried out under certain conditions. All experiments were conducted with at least three replicates.

2.5.1. Solvent selection

The 300 μL of solvent was added to 3.2 mm disks of DPS placed in 1.5 mL Eppendorf tube. Samples were incubated on a shaker (TS-100C; BioSan, Latvia) at 800 rpm for 30 min at 30 °C. After centrifugation for 10 s at 1000 g, 250 μL of the solution was transferred to a 300 μL vial for further LC-MS analysis.

2.5.2. Extraction time selection

The extraction was carried out as for solvent selection but with different extraction time: 15 min, 30 min, 45 min, 60 min, 75 min and 90 min.

2.5.3. Extraction temperature selection

The extraction was carried out as for solvent selection but with different extraction temperature: 30 °C, 40 °C, 50 °C, 60 °C.

2.5.4. Solvent volume selection

The different solvent volume: 100 μL, 200 μL, 300 μL, 400 μL, 600 μL and 800 μL was added to 3.2 mm disks of DPS placed in 1.5 mL Eppendorf tube. Samples were incubated on a shaker (TS-100C; BioSan, Latvia) at 800 rpm for 30 min at 30 °C. After centrifugation for 10 s at 1000 g, solutions were transferred to a new Eppendorf tubes. The solvent was evaporated to dryness using Labconco SpeedVac systems (Labconco, USA). Samples were reconstituted in 100 μL of MeOH and transferred to a 300 μL vial for further LC-MS analysis.

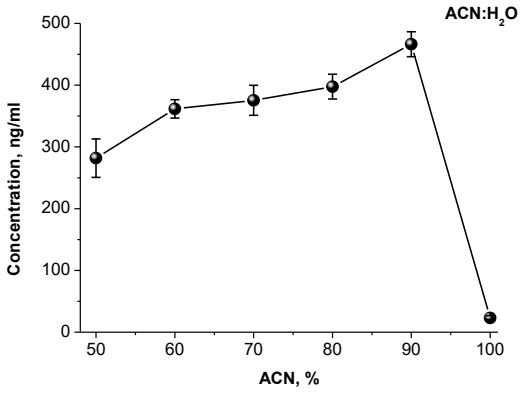
3. RESULTS AND DISCUSSION

The most suitable method for extraction from DPS is organic extraction [15]. It is a one-step process that simply adds an organic solvent directly to the samples in the DPS. With this approach, red blood cells and proteins stay inside the spot, and the target substance is retrieved into a solvent. For further use of LC-MS analysis, methanol and acetonitrile are best suited as solvents.

The first step in this work was to select the solvent that provides the greatest recovery, since there is no data on apixaban extraction from DPS, but for extraction from DBS, authors used 100% and 70% methanol, 100% and 70% acetonitrile and 0.1% formic acid in 70% acetonitrile in the work [6]. Various types of solvents were prepared, consisting of a mixture of methanol or acetonitrile with water in the presence or absence of 0.1% FA, and various mixtures of MeOH:ACN (table 1).

Table 1. Solvent Composition

MeOH:H_2O, % MeOH	MeOH:H_2O, (0,1% FA), % MeOH	ACN:H_2O, % ACN	ACN:H_2O, (0,1% FA), % ACN	MeOH:ACN, % MeOH	MeOH:ACN, % ACN
100	100	100	100	100	100
90	90	90	90	90	90
80	80	80	80	80	80
70	70	70	70	70	70
60	60	60	60	60	60
50	50	50	50	50	50



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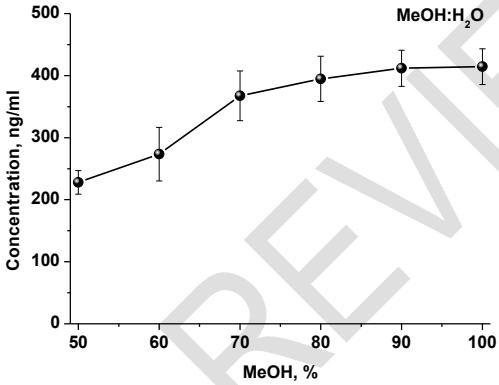

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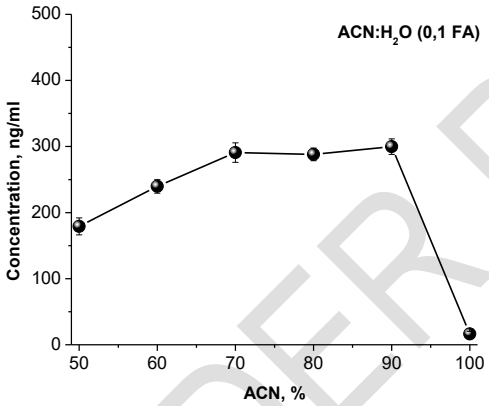

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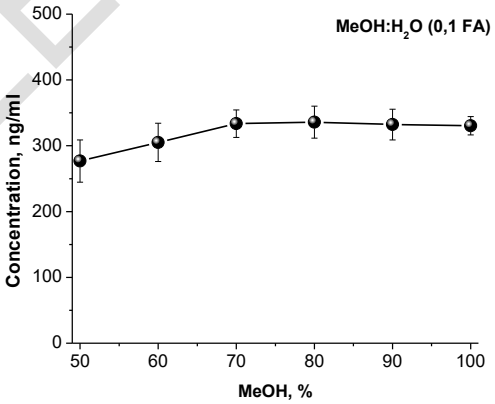

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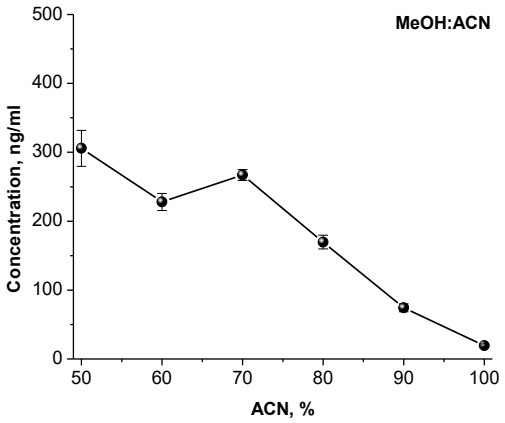

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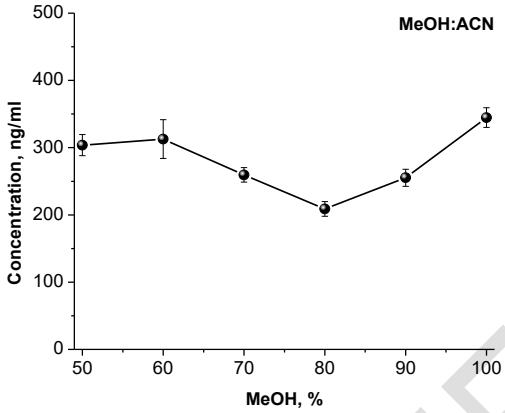

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Figure 2. Apixaban recovery from DPS at the concentration 400 ng/ml by different solvents: ACN:H_2O mixture (a), MeOH:H_2O mixture (b), ACN: 0,1% of FA in H_2O mixture (c), MeOH: 0,1% of FA in H_2O mixture (d), MeOH:ACN mixture (e and f).

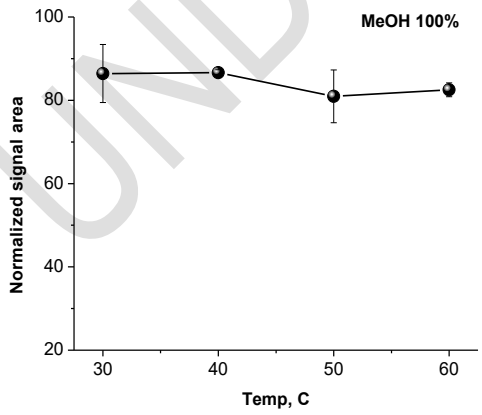
All experiments were performed under the same conditions in three repeats in order to compare the efficiency of apixaban extraction from DPS with solvents. Each sample was analyzed three times by the LC-MS method. The results are shown in Fig. 2.

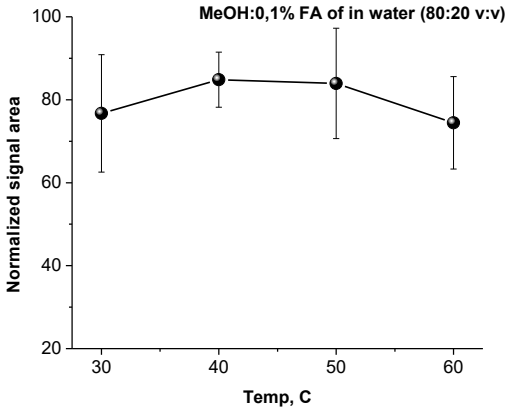
For mixtures of MeOH:H_2O and ACN:H_2O, the increase in apixaban extraction was observed with growth in the percentage of methanol and acetonitrile, respectively, with the exception of 100% acetonitrile (Fig. 2a, 2b). The addition of 0.1% formic acid resulted in about 30% reduced extraction (Fig. 2d, 2c).

In the MeOH:ACN mixture, the highest efficiency was achieved at 100% methanol, and the lowest at 80% (Fig. 2f); the highest efficiency was demonstrated at 50% ACN followed by a decrease in apixaban extraction with a further increase % of acetonitrile (Fig. 2e).

To optimize other extraction parameters, 4 solvents were selected that showed the highest efficiency: 100% MeOH, MeOH: 0,1% FA в H_2O (80:20, v:v), ACN:H_2O (90:10 v:v) and ACN: 0,1% FA в H_2O (90:10, v:v).

The next step was to determine the optimal extraction temperature. Extraction was performed at different temperatures from 30 °C to 60 °C degrees in 10 °C increments for each selected solvent mixture (Fig. 3). Further temperature increases don't make sense, as it lead to evaporation of solvents and loss of solvent volume, resulting in a higher measurement error. The best efficiency is achieved at 40 °C for all solvents. As the temperature increases, the signal level decreases a little.





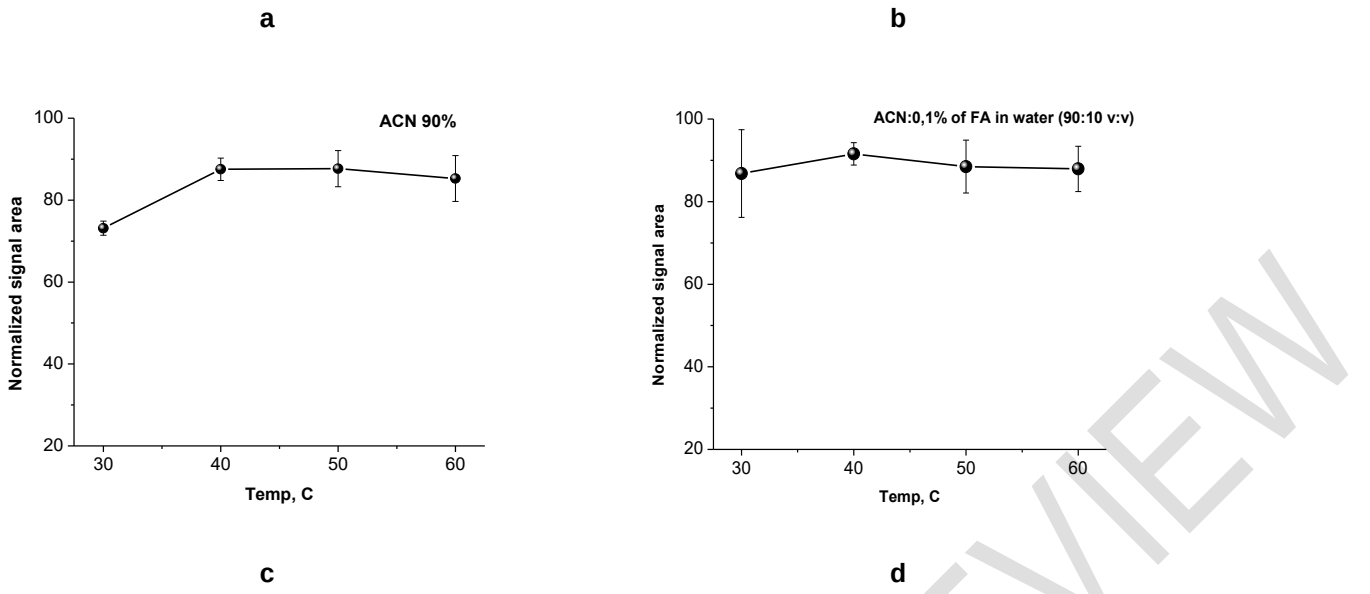


Figure 3. Normalized apixaban recovery from DPS at different temperature: MeOH:H_2O mixture (a), MeOH:0,1% of FA in H_2O mixture (b), ACN:H_2O mixture (c), ACN:0,1% of FA in H_2O mixture (d).

For methanol, there was an increase in extraction efficiency with increasing incubation time of about 5%, and for acetonitrile, a decrease efficiency of about 10% (Fig 4). The 15 minutes will be enough for apixaban extraction in mixtures of acetonitrile and 90 minutes in mixtures of methanol from a 3 mm disk of DPS.

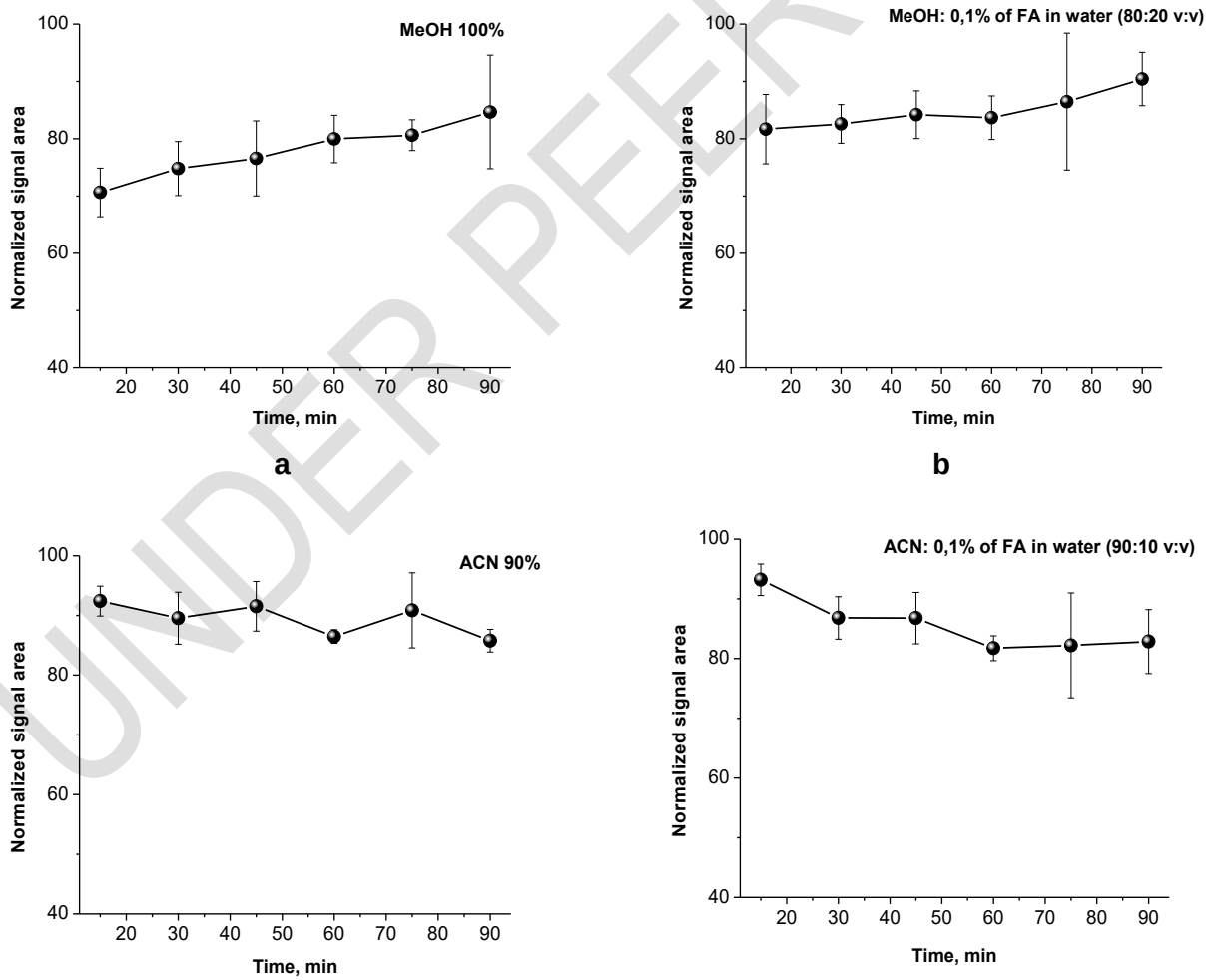


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Figure 4. Normalized atenolol recovery from DPS at different temperature: MeOH:0,1% of FA in H_2O mixture (a), ACN:0,1% of FA in H_2O mixture (b), MeOH:H_2O mixture (c), MeOH:ACN mixture (d).

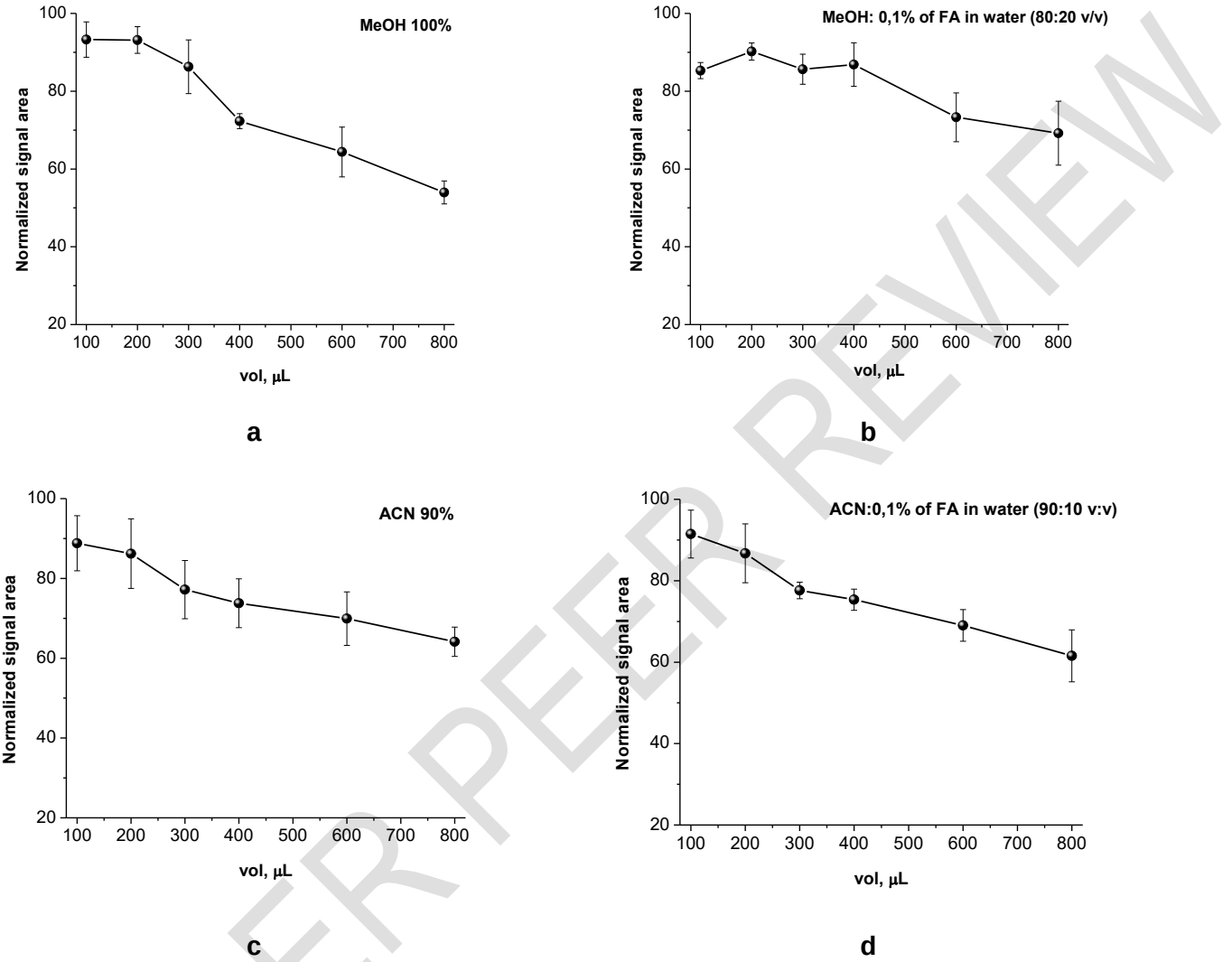

Figure 5. Normalized apixaban recovery from DPS with different solvent volume: MeOH:H_2O mixture (a), MeOH:0,1% of FA in H_2O mixture (b), ACN:H_2O mixture (c), ACN:0,1% of FA in H_2O mixture (d).

Since there was used a single-stage extraction method to compare different volumes of solvent in the work, the samples were evaporated to dry and then were resolved in 100 μL of pure methanol. Otherwise the increase in the volume of the solvent will lead to a decrease in the signal level. With increasing volume, the efficiency of apixaban extraction decreases rapidly. This may be due to the fact that when samples are redissolved in a small amount of solvent, a certain amount of apixaban remains on the walls of the tubes. As seen in Figure 5, the optimal volume is 100 μL for all solvents.

4. CONCLUSION

In this study, the extraction method was optimized for determining apixaban in DPS samples. The method was tested in terms of the dependence of extraction on time, temperature, as well as the volume and type of solvent. It is shown that the optimal extraction parameters are: incubation time - 15 minutes for mixtures of ACN and 90 minutes for mixtures of MeOH, temperature 40 °C, 100 μL of solvent. Subject to further analysis of LC-MS, it is better to use 90% acetonitrile as solvents, since it has shown the most optimal conditions. Pure acetonitrile is not a suitable solvent for extracting apixaban. Adding 0.1% FA to solvent mixtures reduces apixaban extraction from DPS of about 30%, but without reliable

confirmation. For better optimization, additional experiments must be performed with detailed parameterization in the range set in this work.

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