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## **Parameters Optimization of Edoxaban Extraction from Dried Plasma Spots**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author AAC designed the study, performed the statistical analysis. Authors GAZ and AAC managed the analyses of the study. Author GAZ wrote the first draft of the manuscript. Author VVK wrote the protocol and managed the literature searches. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Aims:** Edoxaban is a direct-acting oral anticoagulant, being a highly selective, direct and reversible factor Xa inhibitor. Edoxaban is used to treat and prevent blood clots such as deep vein thrombosis and pulmonary embolism. The dried spot technic, including dried blood spots and dried plasma spots, is used in many fields, from newborn screening to monitoring of therapeutic drugs in toxicology. In this case, equipment with a highly sensitive detector, such as a mass spectrometer, is required, as well as conditions for a high degree of drug recovery from the dried spot. In this work, the extraction of edoxaban from dried plasma spots (DPS) was studied to determine the optimal parameters of the extraction method.

**Study Design:** Analytical experimental study. Short Research Articles.

**Place and Duration of Study:** Core Facility of Mass Spectrometric Analysis, Institute of Chemical Biology and Fundamental Medicine SB RAS, between August and October 2020

**Methodology:** The organic extraction method was selected for evaluation as the most suitable for LC-MS analysis. Several parameters were investigated to find the best combination for extracting

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edoxaban from DPS for further LC-MS analysis: percent organic solvent, presence or absence of 0.1% formic acid (FA), extraction time, volume, and temperature.

**Results:** The results showed that the extraction was influenced by the composition and volume of the solvent, but not temperature and time. Pure acetonitrile is the worst solvent for extracting edoxaban from DPS. The most optimal parameters are MeOH: 0.1% FA in H<sub>2</sub>O (70:30, v:v) solvent with an extraction temperature of 40 °C, an extraction time of 15 minutes and a solvent volume of 50 µl.

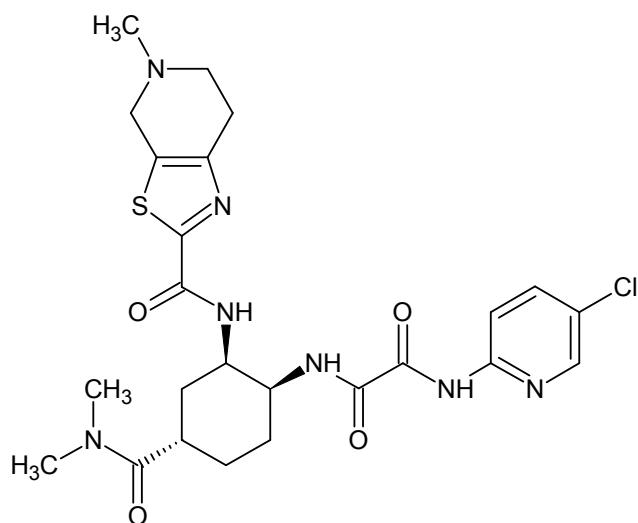
**Conclusion:** Several solvents suitable for LC-MS analysis can be used to recover edoxaban from DPS.

**Keywords:** *Dried plasma spot; DPS; DBS; edoxaban; LC-MS/MS; extraction.*

## 1. INTRODUCTION

Thrombosis is the most common underlying pathology of the 3 major cardiovascular disorders: ischemic heart disease (acute coronary syndrome), stroke, and venous thromboembolism. Ischemic heart disease and stroke are responsible for one in four deaths worldwide. About half of all stroke deaths were from ischemic stroke, which is caused by thrombosis [1]. For decades, vitamin K antagonists such as warfarin and phenprocoumon have served as oral anticoagulants to treat and prevent thromboembolic disorders [2]. Although older generation anticoagulants are effective in preventing thromboembolic complications in atrial fibrillation, they are not as safe - deviations from their narrow therapeutic window can lead to bleeding due to excessive anticoagulation or thrombosis due to insufficient anticoagulation [3]. In addition, there are a number of other

disadvantages such as drug interactions with food and even with viral diseases, as well as with a relatively slow onset actions [4]. Direct oral anticoagulants such as edoxaban can replace vitamin K antagonists because the safety profile of direct oral coagulants is better than that of vitamin K antagonists [3]. Edoxaban highly selectively reversibly inhibit activated factor X [5]. Edoxaban inhibits both free factor Xa and prothrombinase activity. Inhibition of factor Xa in the coagulation cascade reduces thrombin production, prolonged the prothrombin time/international normalized ratio, and reduces the risk of thrombus formation [4,6–8]. Edoxaban is indicated for reducing the risk of stroke and systemic embolism in adult patients with nonvalvular atrial fibrillation with one or more risk factors, as well as for the treatment of deep vein thrombosis and pulmonary embolism and prevent recurrence [8]. The chemical structure of edoxaban is presented at Fig. 1.



**Fig. 1. Chemical structure of edoxaban**

In 1963, Guthrie R. and Susie A. reported the dry blood spot method as an alternative method of collecting samples for pediatric purposes [9]. Dried blood spots (DBS) are used for many applications, from newborn screening to therapeutic drug monitoring in toxicology [10–13]. Widespread use of dry blood spots is associated with the advantage sampling methods. Since DBS is most often obtained with a finger injection, the fence can be carried out by the patient himself, elimination of the need for a phlebotomy. Besides, a small sample volume is useful if it is intended for use in pediatric or anemic population. Uninfected character DBS and the fact that the dried matrix increases the analyte content stability creates fewer difficulties in terms of transportation and storage. In addition, the sample preparation procedure also simple and amenable to automation [10]. 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 are works [11, 14], in which the authors used an internal standard with LC-MS / MS method to assess the concentration of edoxaban in DBS, plasma, whole blood, but there are no studies for the quantitative determination of edoxaban in dry plasma spot. One of the first steps in determining the concentration of edoxaban is to extract it from the DPS. Thus, the aim of this study was to find the optimal parameters for the extraction of edoxaban from DPS.

## 2. MATERIAL AND METHODS

### 2.1 Reagents

Edoxaban, Whatman 903 Protein Saver Card and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile of LC-MS grade were purchased from Panreac AppliChem (Barcelona, Spain). Methanol of HPLC grade were purchased from J. T. Baker (Gliwice, Poland). 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). An analytical column EcoNova ProntoSil-120-3-C18 (2 × 75 mm, 3 µm) (EcoNova, Russia) was used in the chromatograph. Sample injection volume was 10 µL. The flow rate was 0.2 mL/min and the eluent 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 5 min. The autosampler temperature was held at 6°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 340°C. The nebulizer gas pressure and flow were 40 psi and 7 L/min, respectively. Dwell time was set to 200 ms. The ion transitions for edoxaban were m/z 548.2→366.2 (collision energy 20 V, fragmentor voltage 135 V) as a quantifier; m/z 548.2→349.2 (collision energy 25 V, fragmentor voltage 135 V) and m/z 548.2→152.1 (collision energy 35 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. Typical LC-MS/MS chromatogram of edoxaban is presented in Fig. 2.

### 2.3 Preparation of Calibration Standard

For preparation of calibration standards, working stocks of edoxaban at concentrations of 10000 ng/mL were arranged. The calibration standard contained 9 final concentrations of 1, 2.5, 5, 10, 25, 50, 100, 250 and 500 ng/mL. This calibration standard was used in experiments with five different types of solvents.

The edoxaban stock solution was diluted with acetonitrile to prepare intermediate stock solutions that were added to blank rat plasma to create calibration standards with edoxaban concentrations of 100, 200, 400, 500 and 600 ng/mL. The calibration standards (each consisting of 25 µL of rat plasma) were placed on a Whatman 903 Protein Saver Card (GE Healthcare, USA) to fill the circles on the card and were air dried completely for 12 h. After that, 3.2 mm circles of DPS were cut by means of a DBS Puncher, and each circle was placed in a 1.5-mL Eppendorf tube. This calibration standard was used in experiments with selected solvents.

## 2.4 Preparation of Internal Standard

Apixaban was used as an internal standard for edoxaban. The stock solution of apixaban was diluted with acetonitrile to create an internal standard with an apixaban concentration of 10 ng/ml.

## 2.5 Sample Preparation

Stock solution and working samples were prepared in same way as described in work [15]. Briefly, edoxaban was dissolved in 70% acetonitrile to prepare a 10 mg/mL stock solution. The edoxaban stock solution was diluted with 70% acetonitrile to prepare intermediate stock solution that was added to blank rat plasma to create working solution with edoxaban concentration of 400 ng/mL. All stock solutions were stored at -20°C. All 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 edoxaban 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. These circles were 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.

## 2.6 Solvents Preparation

Since there is no data on edoxaban extraction, the five different types of solvents were chosen and prepared according to work [16]. The first

one: MeOH:H<sub>2</sub>O mixture from 0% to 100% of MeOH (v:v) with 10% step. The second one: MeOH: 0,1% of FA in H<sub>2</sub>O mixture from 0% to 100% of MeOH (v:v) with 10% step. The third one: ACN:H<sub>2</sub>O mixture from 0% to 100% of ACN (v:v) with 10% step. The fourth one: MeOH:0,1% of FA in H<sub>2</sub>O mixture from 0% 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 (Table 1). At the end of the extraction, four solvents with the same concentration were selected, with which further experiments were carried out.

## 2.7 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. Extractions in solvent selection experiments were performed without the addition of an internal standard. In other experiments, extraction was performed with the addition of an internal standard.

### 2.7.1 Solvent selection

The 100 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 15 min at 40°C. After centrifugation for 10 s at 1000 g, the solution was transferred to a 300 µL vial for further LC-MS analysis.

**Table 1. Solvent Composition**

MeOH:H <sub>2</sub> O, % MeOH	MeOH:H <sub>2</sub> O, (0,1% FA), % MeOH	ACN:H <sub>2</sub> O, % ACN	ACN:H <sub>2</sub> O, (0,1% FA), % ACN	MeOH:ACN, % MeOH
100	100	100	100	100
90	90	90	90	90
80	80	80	80	80
70	70	70	70	70
60	60	60	60	60
50	50	50	50	50
40	40	40	40	40
30	30	30	30	30
20	20	20	20	20
10	10	10	10	10
0	0	0	0	0

### 2.7.2 Extraction time selection

The extraction was carried out in the same way as when choosing a solvent, but with the addition of an internal standard with a concentration of 10 ng/mL and with a different extraction time: 15 min, 30 min, 45 min, 60 min, 75 min and 90 min.

### 2.7.3 Extraction temperature selection

The extraction was carried out in the same way as when choosing a solvent, but with the addition of an internal standard with a concentration of 10 ng/ml and with different extraction temperature: 20°C, 30°C, 40°C, 50°C, 60°C.

### 2.7.4 Solvent volume selection

Various solvent volumes: 50 µL, 100 µL, 200 µL, 300 µL, 400 µL and 800 µL, containing an internal standard at 10 ng/mL, were added to 3.2 mm DPS discs placed in 1.5 mL Eppendorf tube. Samples were incubated on a shaker (TS-100C; BioSan, Latvia) at 800 rpm for 15 min at 40 °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 the solvents used to prepare the internal standard and transferred to a 300 µL vial for further LC-MS analysis.

## 2.8 Data Analysis

All the data were processed in the Origin 8.0 software (Statsoft Inc., Tulsa, OK, USA), were reported as mean ± standard deviation (SD).

## 3. RESULTS AND DISCUSSION

Due to LC-MS/MS is the reference method for measurement of plasma edoxaban concentration [6], using this method allow to analyze the small amount of substances. Since only about 25 µL of plasma is required to prepare the DPS, blood collection allow to work with small animals at the different stage of preclinical studies or make it less invasive in case of human.

The most suitable method for extraction from DPS is organic extraction [12]. 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 calibration without adding an internal standard.

An important step in this work was the choice of the solvent providing the highest recovery, since there are no data on the extraction of edoxaban from DPS, but for extraction from DBS, authors used 30:70 isopropanol/methyl t-butyl ether (v/v) in the work [11]. Other authors used precipitation of proteins with acetonitrile for extraction [14]. 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).

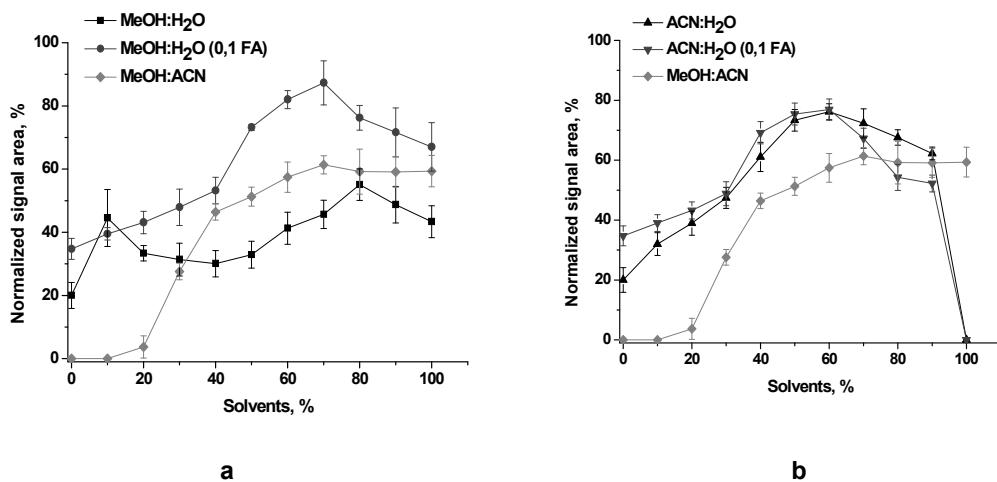
All experiments were performed under the same conditions in four replicates to compare the efficiency of the extraction of edoxaban from DPS using solvents. Each sample was analyzed twice using LC-MS. The results are shown in Fig. 2.

For a MeOH:H<sub>2</sub>O mixture, an increase in extraction was observed at 50% of methanol and a decrease after 70% of methanol.

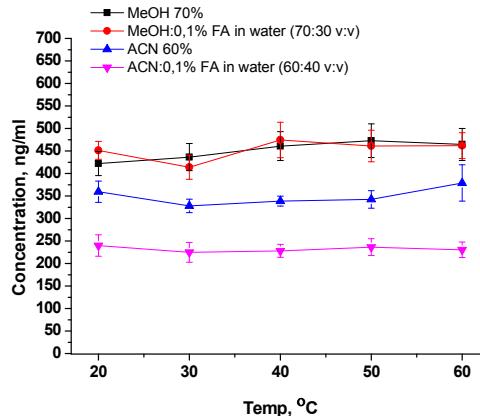
For the ACN:H<sub>2</sub>O mixture, an increase in extraction was observed at 40% of acetonitrile and a decrease after 60% of acetonitrile. The addition of 0.1% formic acid to the ACN:H<sub>2</sub>O mixture resulted in a slight increase in extraction. The addition of 0.1% formic acid to the MeOH:H<sub>2</sub>O mixture increased the extraction by about 20%. In the MeOH:ACN mixture, the highest efficiency was achieved at 70% of methanol and the lowest at 0%; with an increase in the concentration of acetonitrile in the MeOH:ACN mixture, the extraction efficiency decreases (Fig. 2).

To optimize other extraction parameters, 4 solvents were selected that showed the highest efficiency: MeOH (70:30 v:v), MeOH: 0,1% FA in H<sub>2</sub>O (70:30, v:v), ACN:H<sub>2</sub>O (60:40 v:v) and ACN: 0,1% FA in H<sub>2</sub>O (60:40, v:v).

The next step was to determine the optimal extraction temperature. Extraction was performed at different temperatures from 20 °C to 60 °C degrees in 10°C increments for each selected solvent mixture. Further temperature increases don't make sense, as it leads to evaporation of solvents and loss of solvent volume, resulting in a higher measurement error. It was shown that temperature don't affects the extraction efficiency in the range 20-60°C (Fig. 3).



**Fig. 2. Normalized edoxaban recovery from DPS by different solvents: MeOH:H<sub>2</sub>O mixture, MeOH: 0.1% of FA in H<sub>2</sub>O mixture, MeOH:ACN mixture (a), ACN:H<sub>2</sub>O mixture, ACN: 0.1% of FA in H<sub>2</sub>O mixture, MeOH:ACN mixture (b)**

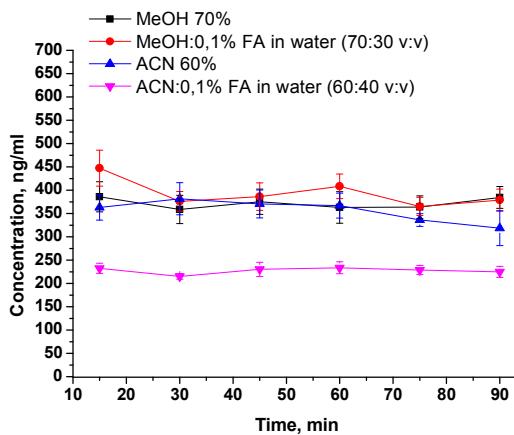


**Fig. 3. Edoxaban recovery from DPS at the concentration 400 ng/ml at different temperature: MeOH:H<sub>2</sub>O mixture, MeOH:0.1% of FA in H<sub>2</sub>O mixture, ACN:H<sub>2</sub>O mixture, ACN:0.1% of FA in H<sub>2</sub>O mixture**

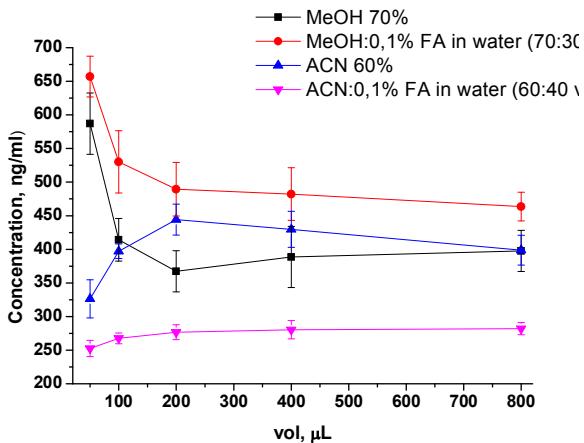
The next experiment was to determine the optimal extraction time (Fig 4). The extraction was carried out under the same conditions, except for the change in the incubation time from 15 to 90 minutes in 15 minute increments for each selected solvent mixture. For methanol and 60% acetonitrile with addition of formic acid, the optimum extraction time was 15 minutes. For 60% acetonitrile, the optimum extraction time was 30 minutes. With increasing incubation time, no significant changes in extraction were observed.

Since in this work, to compare various volumes of the solvent, we used the one-stage extraction

method, the samples were evaporated to dryness and then dissolved in 100 µL of the solvent with which they were extracted (Fig 5). Otherwise, an increase in the volume of solvent will lead to a decrease in the signal level. As the volume of solvent increases, the extraction of edoxaban with methanol decreases. In this case, the most efficient extraction volume was 50 µL. In the case of acetonitrile, the optimal extraction volume was 200 µL. With a further increase in the volume of 70% acetonitrile, the recovery decreases, but in presence of 0.1% formic acid, the extraction efficiency does not change.



**Fig. 4. Edoxaban recovery from DPS at the concentration 400 ng/ml at different time: MeOH:0,1% of FA in H<sub>2</sub>O mixture, ACN:0,1% of FA in H<sub>2</sub>O mixture, MeOH:H<sub>2</sub>O mixture, MeOH:ACN mixture**



**Fig. 5. Edoxaban recovery from DPS at the concentration 400 ng/ml with different solvent volume: MeOH:H<sub>2</sub>O mixture, MeOH:0,1% of FA in H<sub>2</sub>O mixture, ACN:H<sub>2</sub>O mixture, ACN:0,1% of FA in H<sub>2</sub>O mixture**

#### 4. CONCLUSION

In this study, the extraction method was optimized for determining edoxaban 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 MeOH and ACN: 0.1% FA in H<sub>2</sub>O (60:40 v:v), 30 minutes for ACN:H<sub>2</sub>O (60:40 v:v); temperature 40 °C for mixtures of MeOH and 20 °C for mixtures of ACN; volume 50 μL for the mixture of methanol and 200 μL for the mixture of acetonitrile. Subject to further analysis by LC-MS, it is better to use 70% methanol containing

0.1% formic acid as solvents, since it provide the best extraction. Pure acetonitrile is not a suitable solvent for extracting edoxaban. The adding of 0.1% formic acid to the solvent mixture affects them ambiguously. In the case of acetonitrile, no significant change in the extraction efficiency was observed. The adding of 0.1% formic acid to methanol increases the extraction efficiency by 20%. For better optimization, additional experiments must be performed with detailed parameterization in the range set in this work.

#### CONSENT

It is not applicable.

## ETHICAL APPROVAL

As per international standard or university standard written animal ethical approval has been collected and preserved by the author(s).

## DISCLAIMER

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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