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ORIGINAL ARTICLE

# Analytical methodology and pharmacokinetic study of elagolix in plasma of rats using a newly developed UPLC-MS/MS assay

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**KEYWORDS**

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**Abstract** Elagolix, as a competitive gonadotropin-releasing hormone (GnRH) receptor antagonist, has been recently approved by the US FDA for the management of moderate to severe pain due to endometriosis in women. In this study, we developed and verified an analysis assay to detect the concentration level of elagolix in plasma from rats after sample preparation based on a newly validated ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) technique in this study. The process of sample preparation used acetonitrile for a quick and easy protein precipitation method and diazepam was engaged as the internal standard (IS). Then, gradient elution was used to elute elagolix and IS. The mobile phase used in the present experiment was consisted of solvent A (acetonitrile) and solvent B (water having formic acid with the volume ratio of 0.1%), and the type of the C18 column used was named Acquity UPLC BEH C18 column with the specification of 2.1 mm × 100 mm, 1.7 μm. Multiple reaction monitoring (MRM) in positive ion mode for the experiment was engaged to detect the level of elagolix with electrospray ionization (ESI) source by $m/z$ 632.4 → 529.5 transition for quantification and $m/z$ 632.4 → 177.1 transition for qualification. It was found that the method in the scope of 1–2000 ng/mL indicated excellent linearity ($r^2$ > 0.9983). The precision of this assay for intra-day was between 3.5 and 5.5%, and for inter-day was between 9.4 and 12.7%, respectively; the accuracy was 1.2–13.9% for the intra- and inter-day. The stability, extraction recovery, and matrix effect of the method were all in accordance with the rules of assay validation in biological medium proposed by FDA, whose

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application was also successfully used to determine the concentration of plasma elagolix from an experiment on pharmacokinetic investigation after oral administration of 15 mg/kg elagolix.



## 1. Introduction

As one of the common diseases in gynecology, uterine fibroid is a common estrogen-responsive neoplasms and noncancerous neoplasms of the uterus, which may cause complications such as anemia and infertility. About 50% of patients with uterine fibroid have obvious clinical manifestations, and the symptoms of original site usually include heavy menstrual bleeding and pelvic pain. Symptoms other than the reproductive system symptoms include urinary and gastrointestinal symptoms (Baird et al., 2003; Stewart, 2001; Stewart, et al., 2017). The incidence of endometriosis in women of reproductive age is about 10%, and it is a common estrogen-dependent disease in gynecology(Giudice and Kao, 2004). The reproductive system manifestations include pelvic pain, abnormal uterine bleeding and abnormal ovulation, some other patients will also have symptoms such as painful urination(Giudice, 2010; Johnson et al., 2013).

Endometriosis, uterine fibroid and their related symptoms may have a major impact on women's psychological and overall health and their quality of life, which can also bring a huge economic burden to society (Fuldeore and Soliman, 2017; Vercellini et al., 2011). Although the mechanism of the clinical manifestations of uterine fibroids and endometriosis was not clear, the treatment of menstrual bleeding and pain associated with them related to estrogen. According to two clinical researches published in New England Journal of Medicine (NEJM) (Schlaff et al., 2020; Taylor et al., 2017), oral gonadotropin-releasing hormone (GnRH) receptor antagonists can reduce the occurrence of these two symptoms or make them less severe by inhibiting ovarian sex hormones.

Elagolix (Fig. 1), as an orally bioavailable, nonpeptide and competitive GnRH receptor antagonist, is a potential treatment of heavy menstrual bleeding related to uterine fibroid and pain caused by endometriosis currently under development (Ali and Al Hendy, 2021; Ezzati and Carr, 2015; Perricos and Wenzl, 2017; Simon et al., 2020). For the management of endometriosis-associated pain in women, an acceptable efficacy and safety profile of elagolix have been showed in results of phase II and phase III trials (Carr et al., 2014; Diamond et al., 2014; Surrey et al., 2018; Taylor et al., 2017). So, it has been recently approved by the US FDA for the management of moderate to severe pain due to endometriosis in women (Lamb, 2018). The doses of elagolix were evaluated at a once-daily 150 mg or 200 mg twice daily, and it was observed that the suppression of estradiol production is dose-dependent. The absorption and metabolism of elagolix is rapid when administered orally, and the excretion of elagolix in the urine was only 3% dose of oral administererd (Struthers et al., 2009). The time to maximal concentration ($T_{max}$) and the elimination half-life ($t_{1/2}$) is approximate 1.0–1.5 h and 4–6 h, respectively (Ng et al., 2017).

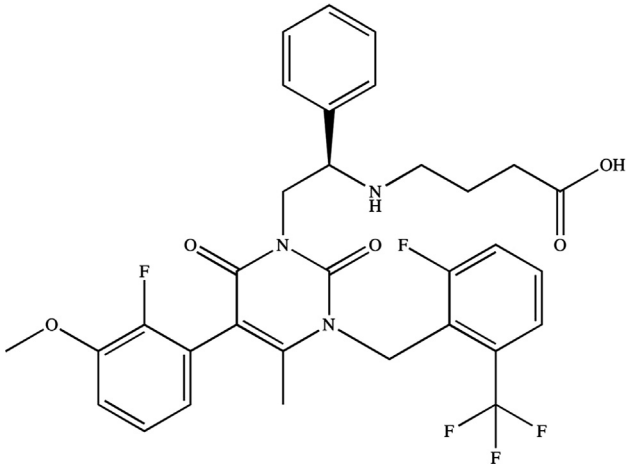


**Fig. 1** The chemical structure of elagolix in the present research.

According to the document retrieval, there is only one published paper to systematically determine the level of elagolix in biological medium (Winzenborg et al., 2018). However, it did not provide enough data to repeat the details of the experiment (e.g. the process of plasma samples preparation, parameters of mass spectrometry, chromatography conditions, and so on). Therefore, it plays an important role to design a reproducible and precise approach to characterize the characteristics of pharmacokinetics on elagolix in rat plasma (Polepally et al., 2020). In the present research, we established and verified an analysis approach for the detection of elagolix in rat plasma based on a newly validated ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) assay. And, we employed the plasma samples of rats after taken by mouth of elagolix at the dose of 15 mg/kg to assess the profiles of pharmacokinetics on elagolix in vivo in our research, which was reported as well.

## 2. Materials and methods

### 2.1. Chemicals

Elagolix (purity >98%) was used as the analyte, and diazepam (purity >98%) was used as the internal standard compound (IS), were provided by Beijing Sunflower and Technology Development CO., LTD (Beijing, China). Merck Company (Darmstadt, Germany) provided methanol and acetonitrile, which were used as HPLC grade. Milli-Q water purification system (Millipore, Bedford, USA) with academic reagent grade was applied to produce deionized water.

### 2.2. UPLC-MS/MS conditions

The chromatographic separation of the analyte and IS used for the analysis was set at 40 °C on an 2.1 mm × 100 mm, 1.7 μm Acquity BEH C18 column, which was a part of the system of Acquity ultra-high performance liquid chromatography

(UPLC) (Waters Corp., Milford, MA, USA). Solution of acetonitrile (A) as organic phase and solution of 0.1% formic acid in water (B) as water phase composed the solvents of the gradient elution. The following method was the gradient program: 10–10% A at the time of 0–0.5 min, 10–90% A at the time of 0.5–1.0 min, 90–90% A at the time of 1.0–2.0 min, 90–10% A at the time of 2.0–2.1 min, and 10–10% A at the time of 2.1–3.0 min. The flow speed of this method was carried out at 0.30 mL/min with 3.0 min of each sample for the total analysis time.

In the detection of the analyte and IS, we used a triple quadrupole mass spectrometer with the pattern of XEVO TQ-S, which was equipped with an electrospray ionization (ESI) source, to perform the mass spectrometric detection under the mode of positive ionization. Besides, we also used multiple reaction monitoring (MRM) to measure the analyte with transitions of $m/z$ 632.4 → 529.5 for quantification and $m/z$ 632.4 → 177.1 for qualification on elagolix and transitions of $m/z$ 285.0 → 154.0 for IS. The data acquirement and instrument control were conducted on the software of Masslynx 4.1, which was equipped with the UPLC-MS/MS system.

### 2.3. Calibration standards and quality control (QC)

1.0 mg/mL methanol for each analyte was used to prepare stock solutions of elagolix and IS. Calibration standard working solutions at a series of concentrations were sequentially diluted from the corresponding working solutions with methanol, which were also obtained from the stock solutions. The dilution of stock solution with methanol was used to prepare the 50 ng/mL IS working solution as well. We added 10 μL of the corresponding working solutions to 90 μL of unadministered blank plasma of rat to prepare the calibration curve (the levels from high to low were 2000, 1000, 500, 100, 50, 10, 5, 1 ng/mL, respectively) and QC rat samples (the concentrations were 2, 800, 1600 ng/mL, respectively). Freezer set at 4 °C was used to store all solutions (including stock solutions and working solutions), and 10 min was needed before experiments we placed the solutions at room temperature.

### 2.4. Sample preparation

In this study we used protein precipitation to prepare samples. Briefly, in a 2.0 mL Eppendorf tube, adding 20 μL IS working solution into 100 μL of plasma, and vortexing the mixture for 30 s. Precipitating the plasma with 300 μL acetonitrile before vortexed it for 2.0 min, then centrifugating it for 10 min at 13,000 × g. And, the UPLC-MS/MS system was applied to carry out the measurement of the supernatant (1 μL).

### 2.5. Method validation

The bioanalytical assay validation of the approach in this study was completely based on the principles of the China Food and Drug Administration, and also validated according to the guidelines of the United States Food and Drug Administration (FDA), as well as the European Medicines Agency (Tang et al., 2020; Xu et al., 2019).

Blank samples from rat plasma were chosen from 6 various batchs of rats, and typical chromatograms of them were analyzed at the corresponding holding times of elagolix and IS to check for endogenous interference, in order to evaluate the selectivity of this assay.

On the calibration curve, y means the ratio of the peak area of elagolix to the IS, x depicts for the notional concentrations, and $1/x^2$ was used as the weight factor stands for reciprocal of the concentration. In addition, the lowest concentration in the calibration curve was considered to be the lower limit of quantitation (LLOQ), with S/N > 10, and relative standard deviation (RSD, %) stands for precision should be lower than 20%, relative error (RE, %) stands for accuracy should be within ±20%.

We compared the peak areas at the respective concentration levels of the post-spiked samples with untreated samples, which were dissolved in methanol, in order to investigate the effect of matrix effect in this method. To estimate extraction recovery, the ratio of the concentration of the analyte added to blank rat plasma before and after extraction, was calculated. Experiments was repeated six times at low, medium, high three different QC levels to determine the extraction recovery and matrix effects.

In addition, we repeated the determination of QC samples of each concentration on three separate days eighteen replicates, a day six replicates during a single analytical run, in order to calculate the precision (RSD%) and accuracy (RE %) for intra-day and inter-day, respectively.

In this study, we analyzed 5 duplicates of samples from rat plasma, in which the levels of elagolix were 2, 800 and 1600 ng/mL respectively, in order to evaluate their stabilities. Freeze/thaw three times, and storage at 10 °C, ambient temperature and −20 °C for the corresponding times 3 h, 2 h, and 28 days respectively were included at the four different possible conditions in this experiment, under which the stability study of elagolix samples at three QC levels were determined.

### 2.6. Pharmacokinetic application

This study adhered to the rules and regulations in regards to the Care and Use of Laboratory Animals of Wenzhou Medical University, which provided the female Sprague-Dawley (SD) rats, whose weights were at 200 ± 20 g. Their food and water intake were not restricted before the experiments. 15 mg/kg elagolix as a single dose for each rat were orally administrated, and at 0, 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 9, 12, 24, and 36 h after administration, we collected 300 μL of blood from each rat into EP tubes with heparin as the anticoagulant. Plasma samples were separated under centrifugation for 8 min at 4000$g$ immediately after collection and kept at −20 °C until later use. The data of key pharmacokinetic parameters of elagolix in rats was evaluated by Drug and statistics (DAS) Version 3.0 software, which was from Shanghai University of Traditional Chinese Medicine, China.

## 3. Results and discussion

### 3.1. Method validation and improvement

At current experiment, we validated an analytical method on elagolix separation from the biological medium of rat plasma through an accurate UPLC-MS/MS assay, in which we optimized the chromatographic conditions to shorten the run time and improve peak shape and detection sensitivity. To improve

the accuracy and reduce the experimental error, we used diazepam as the IS. We selected acetonitrile as the organic phase because it was better than methanol in terms of lower background noise and higher responses. Finally, we obtained good isolation and peak shapes, with 0.1% aqueous solution of formic acid and acetonitrile as mobile phase.

In order to remove protein and potential interferences, an efficient clean-up for preparing samples played a critical role prior to UPLC-MS/MS analysis. In terms of extraction technique in the present method, we used a simple and effective protein precipitation approach instead of complex solid phase extraction and liquid–liquid extraction techniques reported in other papers (Gu et al., 2018; Ocque et al., 2018). We tested various solvents for protein precipitation, such as perchloric acid (6%), trichloroacetic acid (10%), acetonitrile and methanol, and acetonitrile was finally chosen because of its better recovery.

### 3.2. Selectivity and ME

Fig. 2, which confirmed the selectivity of the method, shows the typical chromatograms of drug-free rat plasma samples,

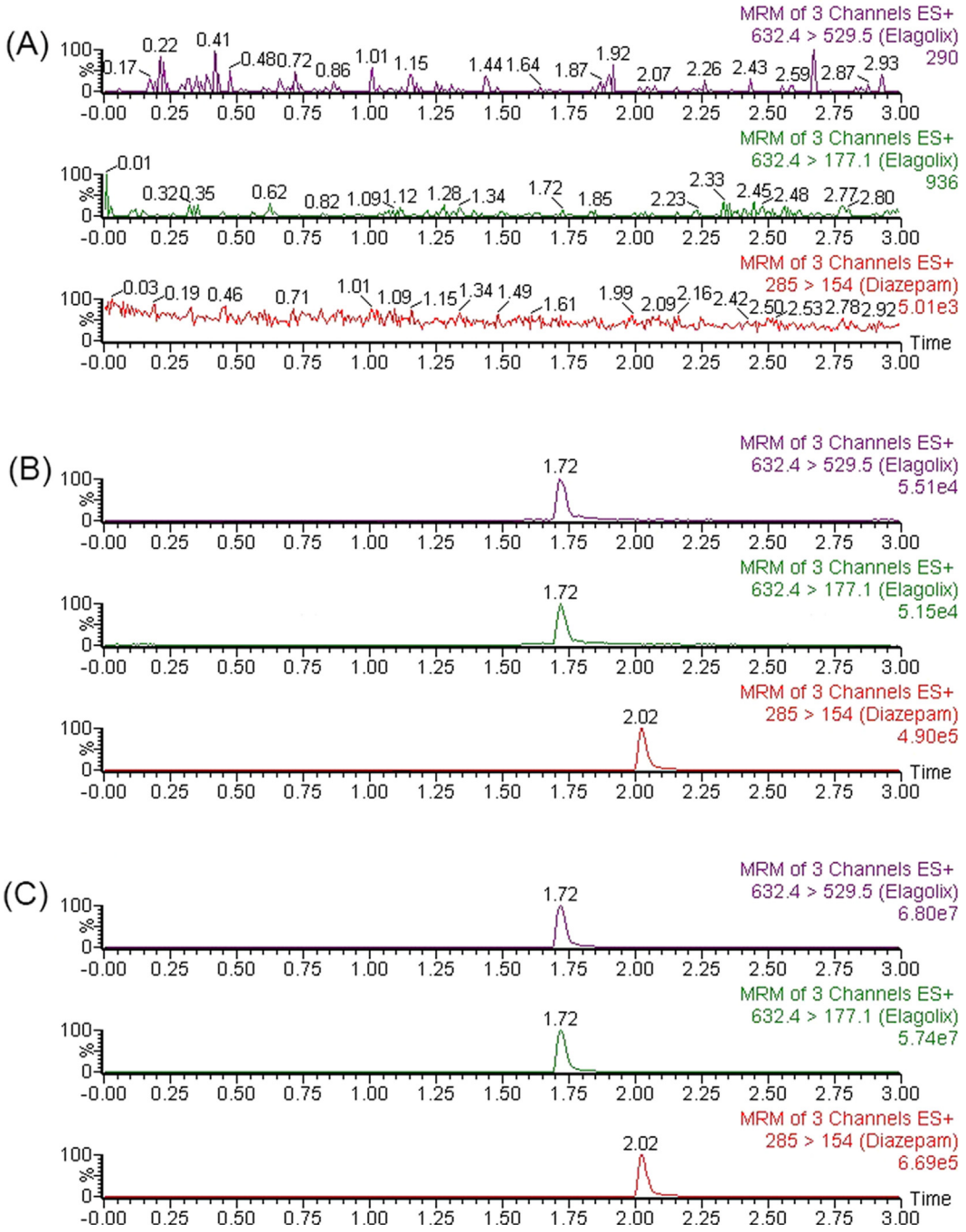


**Fig. 2** Representative chromatograms of elagolix and IS in rat plasma samples. (A) a blank plasma sample; (B) a blank plasma sample spiked with elagolix and IS; (C) a rat plasma sample after oral administration of 15 mg/kg elagolix.

**Table 1** Accuracy, precision, and recovery of QC samples of elagolix from plasma in rats (n = 6).

| Concentration (ng/mL) | RSD (%) | | RE (%) | | Recovery (%) |
|---|---|---|---|---|---|
| | intra-day | inter-day | intra-day | inter-day | |
| 2 | 5.5 | 12.7 | 9.2 | 13.8 | 95.4 |
| 800 | 3.5 | 9.4 | 1.2 | 12.0 | 88.3 |
| 1600 | 4.3 | 10.8 | 11.8 | 13.9 | 89.9 |

**Table 2** Stability findings of elagolix from plasma in rats under different conditions (n = 5).

| Concentration (ng/mL) | room temperature, 2 h | | 10 °C, 3 h | | Three freeze–thaw | | −20 °C, 28 days | |
|---|---|---|---|---|---|---|---|---|
| | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) |
| 2 | 11.9 | 12.4 | 12.6 | 9.3 | 11.6 | 7.4 | 12.1 | −4.1 |
| 800 | 6.8 | 14.2 | 14.8 | 6.3 | 13.2 | 14.3 | 9.0 | 2.9 |
| 1600 | 7.8 | 10.1 | 3.3 | 14.5 | 6.2 | 14.6 | 5.7 | 14.7 |

drug-free rat plasma samples with elagolix and IS added, as well as plasma samples of rats following orally administered elagolix from the pharmacokinetic study. The results of the chromatograms exhibited that the holding time of IS was 2.02 min and elagolix was 1.72 min, respectively, and interfering peaks from endogenous substances were not significant and could be ignored. In plasma, the influence of matrix effect from plasma on the determination of elagolix could be acceptable as the matrix effect of elagolix was found to be 86.1 ± 10.2%, 105.0 ± 9.8% and 97.8 ± 14.1% at 2, 800, and 1600 ng/mL levels, respectively.

### 3.3. Linearity and sensitivity

The typical formula of the calibration standard in our experiment, which was calculated using the ratio of response of elagolix to the IS (y) against the plasma level of elagolix (x), was y = 2964.16x + 1669.53. Good linearity of this method was shown with the correlation coefficient $r^2$ = 0.9983 in the scope of 1–2000 ng/mL on the calibration standard curve. This method of elagolix was sufficient for quantification due to the LLOQ was 1 ng/mL with acceptable accuracy and precision.

### 3.4. Accuracy, precision, and extraction recovery

Six repeated determinations under three different concentrations including 2800 and 1600 ng/mL of QC samples were performed to assess the recovery, precision, and accuracy, and the data was all shown in Table 1. At three QC levels, the recovery of elagolix in rat plasma ranged from 88.3% to 95.4%. Three different days required for the determination of the precision and accuracy were all met the requirements.

### 3.5. Stability

We tested the stability study in four variable possible measurement environment, including the stability of short period of time set at ambient temperature for more than 2 h, the stability of long period of time set at −20 °C for 28 days, and stored in the autosampler (10 °C) for 3 h after extraction, and 3 cycles of complete freeze–thaw study was studied as well. Excellent stability was displayed under all four conditions, and the conclusions of the stability researchs were shown in Table 2.

**Table 3** Main pharmacokinetic parameters after taking a single oral dose of elagolix to rats (n = 6).

| Parameters | Mean ± SD |
|---|---|
| $t_{1/2}$ (h) | 6.56 ± 1.58 |
| $C_{max}$ (ng/mL) | 1515.02 ± 363.19 |
| $T_{max}$ (h) | 1.50 ± 0.23 |
| CL (L/h kg) | 2.87 ± 0.39 |
| $AUC_{0\rightarrow t}$ (ng/mL h) | 5212.98 ± 617.65 |
| $AUC_{0\rightarrow \infty}$ (ng/mL h) | 5301.28 ± 637.77 |

### 3.6. Pharmacokinetic study in this method for its application

In a preliminary investigation of pharmacokinetic experiment which assessing the level of plasma elagolix concentration after oral administration to the female rats, applicability of the

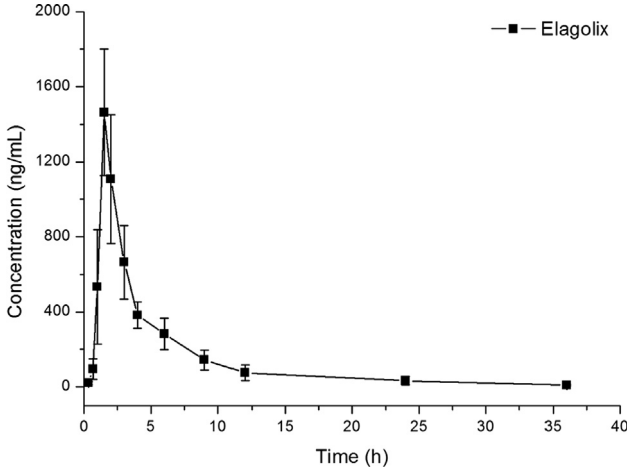


**Fig. 3** Plasma concentration versus time after oral administration of 15 mg/kg elagolix in six rats (Mean ± SD).

method was engaged. The results of the key pharmacokinetic parameters we explored were all shown in Table 3. After taking a single oral dose of elagolix to rats, the relationship between the usual plasma concentration versus time depiction was represented in Fig. 3.

As a result of oral administration of elagolix to rats, the elimination was described by terminal half-life ($t_{1/2}$) of approximately 6.56 h, and total clearance (CL) was 2.87 L/h kg. As the pharmacokinetic study of elagolix were performed in rats with a few, further researches in animals should be done. Moreover, in order to apply this method for clinical application, drug metabolism and pharmacokinetic properties of elagolix need to be further elaborated by additional pharmacokinetic studies in humans.

## 4. Conclusions

As a competitive GnRH receptor antagonist, elagolix shows an acceptable efficacy and safety in the treatment of menstrual bleeding and pain related to estrogen in uterine fibroid and endometriosis. We firstly validated and verified a quantitative method of elagolix in rat plasma in this study, which showed excellent linearity among the scope of 1–2000 ng/mL. And, it was employed to the study of animal pharmacokinetics successfully, in which 15 mg/kg elagolix as a single dose for each rat was orally administrated. In addition, high extraction recovery of elagolix in plasma with no matrix effect was shown in this UPLC-MS/MS method after the protein precipitation with acetonitrile.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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