Quality by Design–Applied Liquid Chromatography-Tandem Mass Spectrometry Determination of Enzalutamide Anti-Prostate Cancer Therapy Drug in Spiked Plasma Samples

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ABSTRACT: This research article presents the Quality by Design (QbD)–finalised conditions for a method that uses liquid chromatography-tandem mass spectrometry for the determination of concentration of enzalutamide (ENZ), an atypical anticancer drug, in a drug formulation and in spiked plasma samples. Critical process attributes (CPA) considered to be the influential parameters in separation, identification, and quantification processes by ultrahigh-performance liquid chromatography-electrospray ionisation-tandem mass spectrometry (UHPLC-ESI-MS/MS) were organic content, buffer strength, pH modifier, flow rate, spray voltage, sheath gas, and auxiliary gas that alter critical analytical attributes, such as retention time (R1) and area (R2). These factors were evaluated first in a factorial design (Taguchi orthogonal array design) and then extensively in a central composite design (CCD) to zero-in on the mobile phase for the quantification of ENZ standard drug and along with its internal standard (ENZIS) in spiked plasma samples and in formulation. Pareto chart from initial factorial design (Taguchi orthogonal array design) model suggested which of the CPA factors should be given the weightage, that is, to be exhaustively analysed in the CCD and response surface analysis. The elaborated parameters proposed by World Health Organization were studied by method validation, ie, selectivity, linearity, accuracy, precision repeatability system-suitability tests, method robustness/ruggedness, sensitivity, and stability. The strategy followed gives an insight on the development of a robust QbD-compliant quantitative UHPLC-ESI-MS/MS method for ENZ drug containing plasma samples (spiked).

KEYWORDS: Enzalutamide, anti-prostate cancer, Quality by Design, response surface analysis, spiked plasma samples, UHPLC-ESI-MS/MS

Introduction

Approach of applying Quality by Design (QbD)-based method3–6 development for analysing the drug initially was approved as an effective second-generation androgen receptor inhibitor. Enzalutamide (ENZ) was earlier known as MDV3100 and now the branded version is ‘Xtandi’, an anti-prostate cancer drug. It blocks the androgen receptor by binding to it and by inhibiting the nuclear translocation and interaction with DNA.7,8 The formal chemical name of ENZ is 4-[3-[4-cyano-3-(trifluoromethyl)phenyl]-5,5-dimethyl-4-oxo-2-sulfanylideneimidazolidin-1-yl]-2-fluoro-N-is 4-[3-

prostate cancer (mCRPC) in a clinical trial. Developed method was validated as per bioanalytical method validation proposed by US Food and Drug Administration (FDA).10,11 Many methods of determination of ENZ in plasma samples of dog, human, and in tissue homogenates were reported.12–18 This reported method discusses about the process of optimising the liquid chromatography (LC)-tandem mass spectrometry (LC-MS/MS) parameters simultaneously in a Taguchi orthogonal array and central composite design (CCD) methods. Combined evaluation reduces the experimental run to greater extent. The developed method was found to be compliant with the acceptable limits for the bioanalytical method development laid by the World Health Organization. Pareto chart from initial factorial design (Taguchi orthogonal array design) model suggested which of the critical process attributes (CPA) factors should be given the weightage, that is, to be exhaustively analysed in the CCD and response surface analysis. An ultrahigh-performance liquid chromatography SIL-20AC HT with 20AD instrument with the octadecylsilica
column (C18) of dimensions (5 µm, 2.1 mm × 50 mm) was used, and selectivity on the column was modified using 2-mM ammonium acetate in acetonitrile (20:80) having 0.1% formic acid as ionic modifier as the mobile phase at a flow rate of 0.6 mL min⁻¹. The spray voltage for the method is maintained at the level predicted by the response analysis. Detection was performed using triple quadrupole MS/MS by multiple reaction monitoring (MRM) via a positive electro spray ionisation (ESI) source spray voltage for the method finalised by design of experiments (DOEs) and response surface analysis is 4750 V. Results clearly showed linearity with $r= .9934$ in the concentration range of 0.2 to 50.0 ng mL⁻¹, an intra- and inter-assay precision of 2.7% and 5.1%, respectively, and recovery studies were found to be between 100.8% and 105.6%. The lower limit of quantification (LLOQ) was 0.078 ng mL⁻¹ in 50 µL of human plasma sample. An intra- and inter-assay precision had %RSD less than 5.0%, and recovery studies were found to be in the acceptable range of 88% to 96%. The LLOQ was 0.078 ng mL⁻¹ in 50 µL of human plasma sample. Enzalutamide was approved in 2012 for the treatment of mCRPC post-docetaxel by the US FDA. The objective of this study was to validate a method for quantification of ENZ in plasma (spiked samples).

**Materials and methods**

High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased from Merck (Chennai, India) and used as such. Analytical-grade ammonium acetate and formic acid were used. Extrapure water was produced in the Quest Life Sciences, Chennai, with the help of MilliPore water purification system (EMD Millipore, Billerica, MA, USA). Standard substances, ENZ and deuterated ENZ (ENZIS), were kindly gifted by Quest Life Sciences. All other chemicals used were of AR grade.

**Instrumentation and its conditions**

Liquid chromatography-tandem mass spectrometry positive ESI of ENZ produced the abundant protonated molecule (MH+) at 466.2 m/z, under positive ionisation conditions and subsequently fragmented in MS/MS mode to the product ion spectra, which had mass-to-charge ratio as 209.2 (Figure 2). Mass spectrometry parameters and collision energies were set as derived from the fractional factorial design (FFD)-optimised method. Quantitative analysis was conducted by MRM at 466.2 to 209.2 m/z for ENZ and its internal standard (IS). Several stationary and mobile phases were tested, whereby a combination of a C18 column and a mobile phase of 2-mM ammonium acetate in water containing 0.1% formic acid and acetonitrile gave the optimum separation and sensitivity towards ENZ.

![Figure 1. Structure of ENZ (enzalutamide).](image)

![Figure 2. A Pareto chart showing effects of influential critical process attributes on first response, Rt (retention time).](image)
ESI-MS/MS of ENZ
Mass spectrometry analysis was performed using a TSQ Quantum Triple Quadrupole Mass Spectrometer (Thermo Scientific, San Jose, CA, USA). The injection volume was 10 µL. The ENZ samples were introduced into the mass spectrometer via the ESI source and analysed in the positive ion mode. The pre-ionised ENZ (M+) samples were detected in the MRM mode using the following transitions: 466.2 to 209.2 m/z for ENZ. The QbD-optimised ESI-MS/MS conditions were as follows: spray voltage: 4750 V; sheath gas: nitrogen, 50 (arbitrary units, bar); auxiliary gas pressure, 10 bar; ion transfer capillary temperature: 380°C; collision gas: Ar; and capillary offset voltage: 35 V. High-performance liquid chromatography was performed using a Shimadzu SIL-20AC HT Autosampler with LC-20AD HPLC Pump; both were controlled by CBM-20A module. Software used to acquire data was LCQUAN 2.5.6, which is a 21 CFR Part 11-compliant solution for method development in LC-MS/MS. For the separation of ENZ from its matrix, a QbD-optimised isocratic mobile phase was used. This comprises 2-mM ammonium acetate having 0.1% formic acid in water (20 parts) – acetonitrile (80 parts) at a flow rate of 0.6 mL min⁻¹. During the experimental run, the column and autosampler tray were preserved at 37°C and 4°C, respectively.13,20

Extraction of ENZ from Plasma
About 100 µL of human plasma samples were spiked with 50 µL each of ENZ and ENZIS (100 ng mL⁻¹). To this, 1.8 mL of extraction mixture (containing acetonitrile [80%] [a protein precipitant] and 2-mM ammonium acetate buffer [adjusted to pH 3.0 using 0.1% formic acid] (20%)) was added and then subjected to vortex mixing followed by centrifugation at 13,500 g. After this, 10 µL was injected directly into the triple quadrupole LC-MS/MS system.20

Preparation of the calibration standard, quality control, and IS stock solutions
Primary stock solutions containing 1 µg mL⁻¹ concentration of ENZ and ENZIS were dissolved in acetonitrile, and serial dilutions of this solution were made in mobile phase to achieve working standard solutions at concentrations of 0.25, 0.5, 0.75, 1.5, 8.5, 12.5, 22.5, 30.0, 40.0, and 50.0 ng mL⁻¹ of both ENZ and ENZIS. Solutions to outline the LLOQ and low quality control (LQC), medium quality control (MQC), high quality control (HQC), and dilution quality control samples were prepared in bulk by diluting the primary stock solution. The IS working solution (1 µg mL⁻¹) was prepared by diluting an aliquot of stock solution (1 mg mL⁻¹) of ENZIS with acetonitrile. All stock solutions were stored in glass bottles at -20°C in the dark.

Sample Preparation
Precipitation of plasma proteins was initiated by adding 200 µL of acetonitrile, and 50 µL of both ENZ and ENZIS solutions was added to 100-µL aliquots of human plasma containing 5 µL of calibration standards. This mixture was vigorously vortexed for 10 minutes and centrifuged at 13,500 g for 10 minutes, after which an aliquot (200 µL) of the supernatant was evaporated to dryness in the stream of nitrogen gas. An aliquot equivalent to 10 µL of this solution was then directly injected into the LC-MS/MS system.

Selection and Optimisation of LC-MS/MS Parameters Using DOE
Experimental design for evaluating the CPA was organic content concentration, spray voltage, sheath gas and auxiliary gas pressure, buffer strength (ammonium acetate), pH modifier (formic acid) concentration, and flow rate. These factors were expected to influence critical analytical attributes (CAA), ie, area (R2) and retention time (Rt) (R1) significantly. Fractional factorial design (Taguchi orthogonal array design) and CCD were considered for finding the critically influencing levels of each factors; this 2-step experimental run will greatly reduce the total experiments at least by 30 for a 7-factor process.22 Factorial design based on Taguchi orthogonal array design was used to determine the main effect without their interaction effects, experiments were run as per the design, and Pareto chart was derived. Based on their percent contribution on the responses (Figures 2 and 4), CAA were further taken in a CCD and optimised by applying response surface analysis, whereas the non-influencing ones were left out.

FFD for Screening Method
The 7 factors that were found to influence responses and the ones that need to be analysed extensively in CCD for their influence on CAA were as follows: spray voltage, set at low level to 4000 V and high level at 5000 V, sheath gas pressure (between 20 and 50 bar), auxiliary gas pressure (between 20 and 50 bar), organic solvent (acetonitrile 70% and 90%), buffer strength of ammonium acetate (2 and 10 mM), pH modifier (formic acid at 0.1% and 0.3%), and flow rate (at 0.3 and 0.6 mL min⁻¹). Critical analytical attributes chosen for analyses were Rt (R1) and area (R2). With the help of Design-Expert trial version 10.0, a statistics program for DOE, factors, and responses was chosen and fed into this program. Based on the generated Taguchi orthogonal array design (8 runs) (Table 1) and their levels (Table 2), experiments were conducted. A factorial model was chosen after aliases were eliminated from the model. Most of the influence comes mainly from spray voltage, acetonitrile content, and with buffer strength, whereas flow rate, formic acid content, sheath gas, and auxiliary gas pressure were having influence only at a contribution % >0.1% on the response. The above parameters were finalised after verifying the perturbation plots (Figures 3 and 5) (significance level was set at .05). The most influencing factors found were acetonitrile with more than 98% and buffer strength by 0.5% for Rt, whereas all other factors were found to influence only by a meagre 0.05%
for Rt (R1). For area (R2), the influential CAA were spray voltage and buffer strength. The flow rate was fixed at 0.6 mL min⁻¹ because the elution time gets prolonged at a flow rate of less than 0.6 mL min⁻¹. Formic acid content was fixed at 0.1%, and sheath gas and auxiliary gas pressures were fixed at 50 and 10 bar, respectively.

CCD and Derringer’s Desirability Function for Response Surface Analysis

Three selected factors (content of organic solvent, spray voltage, and buffer strength) need further exploration in a CCD, a response surface design for improving Rt and area. The generated design is shown in Table 3. The experimental point ranges of the selected factors for the response surface method were the same as for FFD, but a centre level was introduced so as to get the median value of extremes (Table 4). A total of 15 experiments for CCD were conducted and the responses were noted down. Analysis of variance test was performed to select function model and to evaluate significance of factors over responses. The significance level was set at <0.05. The selected function model for Rt (R1) was reduced 2F1, whereas for area (R2), a reduced quadratic model was found to fit significantly. For Rt (R1), most influencing factors were interaction terms of acetonitrile and spray voltage (AB), spray voltage and buffer strength (BC), and the squared terms of spray voltage (B²). For area (R2), influential parameter was spray voltage and interaction term between spray voltage and buffer strength and squared terms of spray voltage. Although none of the other factors show significant influence, which was evident from their P value which was less than <0.05, only spray voltage, buffer strength, and acetonitrile have influence on area. With the above finalised models, 2F1 for Rt and a reduced quadratic one for area (R2), criteria were set to minimise the Rt and maximise the area; this was done by applying Derringer’s desirability method of optimisation. Selected model was used to find the desirable point by applying response surface analysis.

Method Validation: Selectivity, Linearity, Precision, Accuracy, Sensitivity, and Stability Studies

Selectivity of the method was ensured by checking that no interference peaks were observed at the Rt of both ENZ and ENZIS with blank plasma samples. Linearity of the method was validated by injecting 9 calibration samples ranging from lower limit of quality control sample, LQC, MQC, HQC, and one concentration in between each level and the standards injected were as follows 0.25, 0.5, 1.75, 7.5, 12.5, 22.75, 32.5, 40.0, and 50.0 ng mL⁻¹. Peak area was plotted against concentration and regression analysis with weightage of x⁻² was given in finding the regression values. Accuracy and precision were determined by injecting the 3 QC samples, LQC, MQC, and HQC, repeatedly for 6 times and the results were analysed for their %RSD. For accuracy, MQC level sample was spiked at 40%, 80%, and 120% of MQC and analysed by the proposed method. Sensitivity of the method was established by injecting the LLOQ for 6 times, and the %RSD was verified to be in the acceptable limits (20% for LLOQ and 15% for ULOQ [upper limit of quantification]). The stability of the analytes (ENZ and ENZIS) in spiked plasma in different storage conditions was examined by analysing low and high QC samples (n = 3 at each concentration) and compared

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**Table 1.** Taguchi orthogonal array design for optimising liquid chromatography-tandem mass spectrometry conditions for determining enzalutamide in plasma samples in coded levels with retention time as response 1 and area as response 2.

| FACTOR 1 | FACTOR 2 | FACTOR 3 | FACTOR 4 | FACTOR 5 | FACTOR 6 | FACTOR 7 | RESPONSE 1 | RESPONSE 2 |
|----------|----------|----------|----------|----------|----------|----------|------------|------------|
| −1       | −1       | 1        | 1        | 1        | −1       | 1        | 1.508      | 312846     |
| −1       | 1        | −1       | 1        | −1       | 1        | −1       | 1.478      | 490436     |
| −1       | −1       | −1       | −1       | 1        | 1        | 1        | 1.487      | 316092     |
| −1       | 1        | 1        | −1       | −1       | −1       | −1       | 1.513      | 492382     |
| 1        | −1       | 1        | −1       | 1        | 1        | −1       | 1.208      | 320762     |
| 1        | −1       | −1       | −1       | −1       | −1       | −1       | 1.182      | 318786     |
| 1        | 1        | −1       | 1        | −1       | 1        | 1        | 1.175      | 490474     |
| 1        | 1        | 1        | 1        | 1        | −1       | 1        | 1.171      | 485628     |

**Table 2.** Actual levels used in the Taguchi orthogonal array design.

| FACTOR NUMBER/NAME | LOW (−1) | HIGH (1) |
|--------------------|----------|----------|
| 1. Acetonitrile, % | 70       | 90       |
| 2. Spray voltage, V| 4000     | 5000     |
| 3. Buffer strength, mM | 2 | 10 |
| 4. Formic acid, % | 0.1      | 0.3      |
| 5. Flow rate, mL min⁻¹ | 0.3 | 0.6 |
| 6. Sheath gas, bar | 20       | 50       |
| 7. Auxiliary gas, bar | 10  | 20  |
their deviation from the nominal concentration. Bench-top stability was done till the value falls below the acceptable limits. Freeze-thaw stability was determined after 3 freeze-thaw cycles of the QC samples by storing at −70°C for 24 hours, and thawing to room temperature before analysis was performed and it was done for 3 times. Short-term stability was determined after exposure of the QC samples at 4°C for 24 hours for 3 days, whereas long-term stability was done up to 14 days. Autosampler stability too was found out. All the samples would be stored in the freezer at ~20°C, and at intervals specified in the respective stability studies, their concentration was verified with the developed QbD-optimised method. All the results were based on their percent difference from their nominal values7,17 (US FDA, 2001). Figure 9 shows representative LC-MS/MS MRM chromatograms obtained from the analysis of plasma spiked...
Table 3. Coded levels of factors used in central composite design to finalise the parameters of LC-MS/MS for plasma determination of ENZ.

| FACTOR 1 | FACTOR 2 | FACTOR 3 | RESPONSE 1 | RESPONSE 2 |
|----------|----------|----------|------------|------------|
| A: ACETONITRILE, % | B: SPRAY VOLTAGE, V | C: BUFFER STRENGTH, mM | RT, MIN | AREA, UNITS |
| −1.414 | 0 | 0 | 1.582 | 378920 |
| −1 | 1 | 1 | 1.51 | 503094 |
| −1 | −1 | −1 | 1.489 | 314734 |
| 0 | −1.414 | 0 | 1.343 | 323476 |
| 0 | 1.414 | 0 | 1.365 | 600346 |
| 0 | 0 | −1.414 | 1.299 | 460340 |
| 0 | 0 | 1.414 | 1.388 | 456744 |
| 0 | 0 | 0 | 1.338 | 458782 |
| 0 | 0 | 0 | 1.34 | 452348 |
| 0 | 0 | 0 | 1.341 | 454982 |
| 0 | 0 | 0 | 1.335 | 456992 |
| 0 | 0 | 0 | 1.329 | 448902 |
| 1 | 1 | −1 | 1.179 | 603456 |
| 1 | −1 | 1 | 1.201 | 404800 |
| 1.414 | 0 | 0 | 1.092 | 528004 |

Table 4. Actual levels of factors used in central composite design.

| FACTOR | NAME                  | LOW (−1) | CENTRE (0) | HIGH (+1) |
|--------|-----------------------|----------|------------|-----------|
| A      | Acetonitrile, %       | 70       | 80         | 90        |
| B      | Spray voltage, V      | 4000     | 4500       | 5000      |
| C      | Buffer strength, mM   | 2        | 6          | 10        |
with ENZ (1 ng/mL). Sample carryover effects were not observed as 100% acetonitrile was used as needle wash.

**Results and Discussion**

**Method development: screening studies, finalisation of influencing parameters with FD, CCD optimisation, method validation, and application**

Method parameters that were to be extensively optimised by CCD was selected after the investigation of Pareto charts derived from the results of experiments conducted based on FD. The Pareto charts from FD suggested that acetonitrile content negatively influences Rt, evidenced from the Pareto chart and perturbation plot (Figures 2 and 3), and buffer strength needs to be optimised further for their effect on Rt (R1). While spray voltage alone influences the area positively which can be evidenced from the Pareto chart and perturbation plot (Figures 4 and 5) (R2), other parameters such as auxiliary gas and sheath gas pressure, flow rate, and formic acid concentration were not able to influence both the responses which is evident from the perturbation plots of Rt and area (Figures 3 and 5). The MS/MS parameter that has considerable influence on peak area was spray voltage which was taken into the CCD for studying elaborately. The flow rate of the LC parameters does influence the Rt but is not statistically significant enough to be considered for CCD, which is evident from Figure 5. Similar to the flow rate, the formic acid concentration too was not considered for investigation in CCD. Flow rate and formic acid concentration were fixed as follows. Flow rate and the concentration of formic acid were set to 0.6 mL min⁻¹ and 0.1%, respectively. Sheath and auxiliary gas pressures were fixed at 50 and 10 bar, respectively. With the factors being reduced from 7 to 3, CCD was generated and 15 experiments were conducted. The model generated for Rt was a reduced 2F1 model which suggested both the interaction terms between spray voltage and acetonitrile and buffer strength (AB and BC); whereas for area (R2), the model that fit was a reduced quadratic model which suggested interaction term of spray voltage and buffer strength (BC); significant influence of acetonitrile and the squared terms of spray voltage was too found. With the models generated, the study of perturbation plots (Figures 3 and 5) suggested that only Derringer’s desirability function could help identify the solution for the desired levels of responses. This is because acetonitrile influences both the response in an opposite manner. Optimisation criteria were set for the area to be maximised and minimisation of the Rt. In the response surface 3D graph and from the perturbation plot, the area and the Rt were opposed to each other because they were in inverse proportions to the content of organic modifier (acetonitrile) and spray voltage. The factors with the most significant influence on sensitivity were content of organic modifier and spray voltage, whereas buffer strength has comparatively less effect than acetonitrile and spray voltage on area and interaction terms of the main factors could find a place in the model for Rt and area. This is mainly due to nature of mobile phase changes with buffer alters the sensitivity. With the criteria set as to maximise the area and Rt to be ‘in the range’ and strength of buffer to be kept at low level (2 mM), the deposition of salt on capillary electrode gets reduced, thereby lifetime of the instrument increases. With the desirability of 0.937 (Figure 6), the following optimised conditions were considered after analysing the response surface plots (Figures 7 and 8): acetonitrile 80% and 2 mM of ammonium acetate buffer (20%) having 0.1% formic acid flow rate at 0.6 mL min⁻¹. The MS/MS parameters set were as follows: spray voltage 4750 V, sheath gas...
and auxiliary gas pressures were set at 50 and 10 bar, respectively. With these settings predicted, Rt was 1.33 and obtained value was 1.34 (Figure 9). In the case of area, predicted and observed values were very close. Standard calibration curves were linear over an ENZ concentration range of 0.2 to 10 ng mL⁻¹ in human plasma (spiked). Linear regression of these curves resulted in a linear fit (Figure 10) of $y = 0.0615x + 0.000474$ ($r = .9984$) with a weightage of $x^{-2}$. Assay sensitivity was determined by analysing LLOQ samples ($n = 6$) in 3 separate validation batches. The precision values was found to be in the range <3.7% (RSD), and accuracy studies has been found to be between 88% and 95% at 3 QC levels; calculated concentrations at each level are shown in Table 5. The same table shows a summary of intra- and inter-assay precision and accuracy data for QC samples in plasma (spiked) containing ENZ. Results show that these results suggest the acceptable accuracy and precision of the described method. The LLOQ was set at 0.078 ng mL⁻¹ for ENZ using 100 µL of spiked plasma (a representative chromatogram is shown in Figure 9, noting that the signal-to-noise ratio for ENZ is >10 times the baseline at 0.078 ng mL⁻¹). The average extraction recoveries determined at LQC, MQC, and HQC concentrations were between 88% and 95% for ENZ. The developed assay was applied to a laboratory-prepared spiked samples and softgel capsules containing ENZ. The mean concentration and recoveries for them were found to be quite good in agreement with the nominal values and that was with precision % <5. The overall process efficiency of ENZ quantified in spiked plasma was consistent at 3 concentration levels, varying from 81.33% to 104.6%, whereas that of the IS was 92.7%. In addition, this demonstrates that the precipitation of plasma via the addition of acetonitrile can be successfully used to extract ENZ from

![Figure 7. Response surface plot showing the effects of selected factors on Rt (retention time).](image)

![Figure 8. Response surface plot showing the effects of selected factors on area.](image)
spiked plasma. The stability results of ENZ during sample handling for 3 freeze-thaw cycles, short-term temperature storage for 3 days, long-term stability for 15 days, and the stability of the processed samples in the autosampler for 30 hours were evaluated and are shown in Table 6. These sample preparation and storage steps had little effect on the
quantification of LQC and HQC samples. Extracted QC s and calibration standards were allowed to stand at 4°C for 24 hours prior to injection without affecting quantification.

**Conclusions**

A robust, simple, QbD-optimised, sensitive MS/MS method was developed and validated for the determination of ENZ concentration in spiked plasma using a protein precipitation and low pressure gradient elution monitored over MRM mode. This assay provides a linear dynamic range from 0.2 to 10 ng mL⁻¹, leading to an LLOQ of 0.078 ng mL⁻¹ using 100 µL of blank plasma. To our knowledge, this is quite a robust report of an LC-MS/MS method for ENZ quantification in plasma spiked, and the rugged nature of this study proves that

| AMOUNT PRESENT, NG ML⁻¹ | AVERAGE AMOUNT RECOVERED, NG ML⁻¹ | ACCURACY, % RECOVERY | PRECISION, %RSD |
|-------------------------|------------------------------------|----------------------|-----------------|
| 0.685                   | 0.651                              | 95.04                | 1.953           |
| 22.75                   | 20.013                             | 87.97                | 2.634           |
| 39.35                   | 36.788                             | 93.49                | 3.626           |
| 0.685                   | 0.622                              | 90.8                 | 2.037           |
| 22.75                   | 19.838                             | 87.2                 | 1.623           |
| 39.35                   | 36.963                             | 93.93                | 2.137           |

| % LEVEL ADDED TO 22.75, NG ML⁻¹ | AVERAGE AMOUNT RECOVERED, NG ML⁻¹ | ACCURACY, % RECOVERY | %RSD |
|-------------------------------|------------------------------------|----------------------|------|
| 40                            | 27.78                              | 88.19                | 2.703|
| 80                            | 36.053                             | 89.02                | 1.963|
| 120                           | 44.867                             | 90.64                | 2.571|

| STORAGE CONDITION | AVERAGE CONCENTRATION, NG ML⁻¹ | AVERAGE Recovered, % | %RSD |
|-------------------|--------------------------------|----------------------|------|
| Bench-top stability, 11 h | 0.629                          | 91.78                | 2.403|
|                    | 21.148                          | 92.96                | 1.781|
|                    | 35.463                          | 90.12                | 3.377|
| Freeze-thaw stability, 3 cycles | 0.632                          | 92.31                | 3.251|
|                    | 21.01                           | 92.35                | 2.736|
|                    | 37.077                          | 94.22                | 1.748|
| Short-term stability, 2d  | 0.617                           | 90.1                 | 3.483|
|                    | 19.838                          | 87.2                 | 1.623|
|                    | 36.963                          | 93.93                | 2.137|
| Long-term stability, 15 d   | 0.627                           | 91.51                | 1.950|
|                    | 22.707                          | 90.1                 | 4.903|
|                    | 35.515                          | 90.25                | 2.394|
| Autosampler stability, 30h  | 0.621                           | 90.66                | 2.408|
|                    | 4.158                           | 94.19                | 3.214|
|                    | 21.34                           | 91.71                | 3.523|

* n = 6.
the method may be applied for various research and other preclinical studies.

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Author Contributions
ASKS analysed the data and wrote the first draft of the manuscript. RV and SP contributed to the writing of the manuscript. All authors reviewed and approved the final manuscript.

Disclosures and Ethics
As a requirement of publication, author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality, and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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