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Analytical Method Validation of EVT201 and Metabolites (Ro461927 and Ro 18-5528) in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry

Aklima Afroze[1], Ding Li[2*]

[1,2]College of Pharmacy, China Pharmaceutical University-210009, Nanjing, Jiangsu Province, P.R. China

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***Corresponding author:** Ding Li **E-mail:** dinglihg@sina.com, **Mobile:** +8613809047083, **Tel:** 02583271289
College of Pharmacy, China Pharmaceutical University-210009, Nanjing, Jiangsu Province, P.R. China

Abstract **Original Research Article**

A highly sensitive and accurate liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been created and validated for measuring the levels EVT201 and its two metabolites, Ro46-1927 and Ro18-5528, in human plasma. This method was then used to analyze plasma samples from healthy Chinese individuals had taken EVT201 capsules orally, aiming to study the drug's behavior in the body over time (pharmacokinetics). The inter precision was within 75.86%, 98.13%, 98.39%, for EVT201 and 81.11%, 92.18%, 92.42% for Ro46-1927 and 138.39% and 141.58%, for Ro18-5528. The LC-MS/MS method proved to be reliable and precise, making it suitable for investigating EVT201's pharmacokinetics. By applying the validated analytical method, the concentrations EVT201 and its metabolites in human plasma were determined. These data were undergone statistical analysis to assess how EVT201 was processed in the human body following oral administration. In summary, the study employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) to develop and validate an analytical method for quantifying EVT201 and its metabolites in human plasma, ultimately facilitating the evaluation of EVT201's pharmacokinetics in humans.

Keywords: EVT201, Human Plasma, Ro18-5528, Ro46-1927 and LC-MS/MS.

INTRODUCTION

EVT201 could produce lower maximum potency intensity on the GABAA receptor [1]. This mechanism action EVT201 can not only activate the GABAA receptor, but also rapidly inhibit the nervous system, and could also avoid excessive activation the GABAA receptor and deep inhibition the nervous system [2].

At present, Zhejiang Jinxing Pharmaceutical Co., Ltd. cooperates with Evotec to develop EVT201 to treat insomnia disorders in China, which was classified as a Category 1 chemical according to the Drug Registration Administration Measures [3]. In 2015, the Phase II clinical trial EVT201 capsule was carried out, using a multicenter, randomized, double-blind, multi-dose, placebo parallel control design to explore and evaluate the effectiveness and safety on EVT201 capsule in treating insomnia disorders [4] .288 subjects were enrolled, including 70 in the 1.5mg group, 72 in the 2.5mg group, 73 in the 5mg group, and 73 in the placebo group. The results polysomnography (PSG) showed that the total sleep time 1.5mg group, 2.5mg group, and 5mg group was 25.52 minutes, 17.42 minutes, and 22.75 minutes longer than that the placebo group in the first half night after taking the medicine [5].

After taking the EVT201 capsule for two consecutive weeks, subjects in the 2.5mg group and 5mg group also showed that the total sleep time was significantly prolonged on the 13th/14th night, which was 19.30 minutes and 18.15 minutes, respectively. The efficacy 2.5mg and 5mg was comparable, the observed values in the treatment group were notably higher compared to those in the placebo group [6].

In the subjective efficacy evaluation, the subjects, compared with the placebo group, the three dose groups significantly shortened the sleep latency and prolonged the sleep duration. The high-dose group demonstrated superior efficacy when compared to the low-dose group. Take "240-420 minutes of TST before treatment" as the stratification factor to further analyze the domestic Phase II clinical data.

The inclusion criteria TST for the subgroup population are stricter. "240-420 minutes of TST before treatment" could represent the disease characteristics population with proposed indications. Through the post-analysis confidence, the test, it was seen that the effectiveness results obtained based on the sample size subgroups are relatively stable. The results showed that in the population of "240-420 minutes of TST before treatment", the 2.5mg group and the 5mg group had a clear therapeutic effect. Compared with the placebo group, the total sleep time was prolonged by 23.69 minutes and 34.49 minutes respectively [7].

In terms of safety, the EVT201 capsule has good safety and tolerance. Throughout the study, no significant adverse events were reported. The most common adverse reactions were related reactions that affect the nervous system, such as dizziness, sleep respiratory syndrome, decreased blood oxygen saturation, etc. The incidence adverse reactions during the double-blind treatment period in the 2.5mg was significantly lower than that in the 5mg 。

2. MATERIALS AND METHODS

2.1 Matrix

Matrix human plasma (EDTA-K2 as an anticoagulant) was used as a blank matrix we prepared standard curves, quality control, double blank, and zero-point samples. Human whole blood (EDTA-K2 as anticoagulant) was used as a blank whole blood was prepared whole blood stability samples and hemolytic matrix samples.

2.2 Solvents and reagents

All solvents and reagents used in the test were recorded in the original data.

2.3 Analysis method

The analysis process was conducted with strict adherence to the SOP (SOP-LAB 005, SOP-LAB 006) of Nanjing Clinical Tech Laboratories and the method draft Draft 20CTM078V1 or its updated version.

2.4 Method validation

The method's reliability and accuracy, a complete validation was carried out following the guidelines provided in the Chinese Pharmacopoeia (2020).

2.5 Validation of analytical methods

We fully validated this analytical method, additional standard curve point concentrations, stability time points, or validation batches were added, as well as necessary and appropriate method validation content. When reviewing the validated analytical method, it was necessary to perform either complete or partial method validation based on the updated specifications analytical method.

2.5.1 INSPECTION STOCK SOLUTION

The accuracy prepared analyte stock solution, we prepared two stock solutions from two analytes separately weighed. The inspection two stock solutions was repeated six times after proper dilution, and the internal standard was added to the dilution process. Compared the mean peak area ratios of the two stock solutions. The precision and the peak area ratios each stock solution was not exceeded 5.0%. If the difference between the average peak area ratios and the two stock solutions did not exceed ± 5.0%, it was considered that the preparation accuracy solutions was acceptable and the stock solutions were prepared accurately. After passing the inspection, the stock solution finds application in the preparation standard curve samples and quality control samples. The preparation and the quality control sample was separated from the standard curve sample, and the stock solution was prepared separately.Deviation (%)=difference between two stock solution responses/average two stock solution responses × l00%。

2.5.2 Standard Curve Sample

In the method validation the standard curve samples was freshly prepared every day. In three analytical batches we investigated the accuracy and precision, the same batch on the blank matrix was used to prepared standard curve samples at 8 concentration levels (2 replicates for each concentration level).

The lower limit quantification EVT201/Ro 46-1927/Ro 18-5528 was 0.100 ng/mL/0.0300 ng/mL/0.0600 ng/mL

The upper limit quantification EVT201/Ro 46-1927/Ro 18-5528 was 100 ng/mL/30.0 ng/mL/6.00 ng/mL

EVT201 (ng/mL): 0.100 (LLOQ), 0.200, 1.00, 3.00, 10.0, 30.0, 60.0, 100 (ULOQ).
Ro 46-1927 (ng/mL): 0.0300 (LLOQ), 0.0600, 0.300, 1.00, 3.00, 10.0, 20.0, 30.0 (ULOQ).
Ro 18-5528 (ng/mL): 0.0600 (LLOQ), 0.100, 0.200, 0.500, 1.00, 2.00, 4.00, 6.00 (ULOQ).

One set standard curve was injected at the beginning the analysis batch and the remaining set was injected toward the conclusion. In the following cases, the standard curve samples were removed and were not involved in the regression calculation. The deviation calculated value exceeds ± 15.0% (± 20.0% for LLOQ). In the final regression results, six standard curve samples with non-zero concentration levels must meet the requirements, and at least one repeat of LLOQ and ULOQ standard curve samples must meet the requirements. The method validation requires consistency we used the weighting factor 1/x2 for quantification during linear regression to establish the

linear relationship between the theoretical concentration and the response.

2.5.3 QUALITY CONTROL SAMPLES

We Used freshly prepared standard curve samples to quantify freshly prepared or sub-packaged quality control samples. In the subsequent scenarios, the quality control sample was removed from the final calculation due to the chromatographic pre-treatment process, and corresponding records were kept. The final report and the original data explained any data excluded from the calculation. The quality control samples at the following concentration levels were used to the accuracy and precision EVT201 and its metabolites Ro 46-1927 and Ro 18-5528.

EVT201(ng/mL):0.100 (LLOQ QC), 0.250 (LQC), 2.00 (GMQC), 20.0 (MQC), 75.0 (HQC).Ro 46-1927 (ng/mL):0.0300 (LLOQ QC), 0.0800 (LQC), 1.50 (GMQC), 8.00 (MQC), 23.0 (HQC). Ro 18-5528 (ng/mL):0.0600 (LLOQ QC), 0.150 (LQC), 0.300 (GMQC), 1.50 (MQC), 4.50 (HQC). DQC (200 ng/mL/60.0 ng/mL/12.0 ng/mL) diluted quality control sample EVT201/Ro 46-1927/Ro 18-5528 was prepared only when necessary. The diluted quality control sample DQC concentration was adjusted according to the actual situation.

3.1 In Batch Accuracy and Precision

Six duplicate quality control samples (including LQC, GMQC, MQC, HQC, and LLOQ QC) were used to evaluated the intra -batch accuracy and precision. The average measured values each concentration level QC sample deviated within ± 15.0% (except for LLOQ QC, the deviation was not exceeded ± 20.0%). The deviation between quality control samples accounting for at least two-thirds and the total number and their theoretical values did not exceed ± 15.0% (except for LLOQ QC, the deviation was not exceeded ± 20.0%); The deviation between a minimum 50% quality control samples at each concentration level and their theoretical values did not exceed ± 15.0% (except for LLOQ QC, the deviation was not exceeded ± 20.0%). The precision each concentration level QC sample was ≤ 15.0% (except LLOQ QC, the precision was ≤ 20.0%).

3.2 Inter batch Accuracy and Reliability

The accuracy and reliability between batches and the evaluation were conducted by three consecutive batches quality control samples (including LQC, GMQC, MQC, HQC, and LLOQ QC, with six replicates at each concentration level, which were conducted in at least two days). For quality control samples in three analytical batches, the deviation between the average measured value each concentration level quality control sample and its theoretical value was not exceeded ± 15.0% (except for LLOQ QC, the deviation was not exceeded ± 20.0%). The precision concentration level QC sample was ≤ 15.0% (except LLOQ QC, the precision was ≤ 20.0%).

3.3 Selectivity

The substances in the determination analyte and internal standard we prepared a single blank matrix from six different sources, and each matrix has at least three replicates. The internal standard determination we prepared a mixed hemolytic matrix (hemolytic matrix was obtained by adding frozen whole blood to the blank matrix, at a certain proportion of frozen whole blood was 5%, and the proportion frozen whole blood was adjusted according to the actual situation), and each matrix was at least three reconstitutions. the high-fat test substance and internal standard determination we prepared a mixed high-fat matrix (high-fat matrix was obtained by adding a specific proportion of fat emulsion into the blank matrix, the fat content was ≥ 20 mg/mL, and the fat emulsion content was adjusted appropriately according to the actual situation), and each matrix was at least three repetitions. The double-blank samples mentioned above exhibit no significant in the vicinity retention time for both substance and the internal standard. The average response (peak area) and the interference peak for the target substance was not exceeded 20.0% average response sample in the lower limit quantification standard curve.

3.4 Matrix effect

We used six single blank matrices from different sources. The matrix effect at the working concentration internal standard and the minimum, medium, and maximum concentration levels analyte. The double blank samples prepared with blank human plasma obtained from six distinct sources were added with a certain amount analyte and internal standard after pretreatment so that their final concentrations were consistent with the injection concentrations minimum, medium, and maximum concentration QC samples respectively. At the same time, we prepared a pure solution containing a certain amount substance under investigation and the reference standards. The final concentration was consistent with the injection concentration minimum, medium, and maximum-concentration QC samples respectively. Repeated the injection three times at each concentration level. At each concentration level, the total precision the matrix factor normalized by the internal standard was ≤ 15.0%. At all concentration levels, the total precision normalized matrix factor and the internal standard was ≤ 15.0%. The matrix effects (plasma), we prepared quality control samples at minimum and maximum concentration levels with the hemolytic matrix, and added an internal standard working solution for pretreatment according to the analysis method. The deviation between the measured concentration at least two-thirds quality control sample and its theoretical concentration was not more than ± 15.0%, then it was considered that the influence hemolysis was ignored. The frozen whole blood was adjusted according to the

actual situation. We added an internal standard working solution for pretreatment according to the analysis method. The deviation between the measured concentration at least two-thirds quality control sample and its theoretical concentration was not more than ± 15.0%, the high fat was considered negligible. The fat content was adjusted according to the actual situation.

4.1 Residues

After the highest detectable concentration sample was injected to analyzed the double-blank sample, the system residue was evaluated. The matrix effect at the concentration analyte and the internal standard determination analytical method we prepared at least one Carryover ULOQ and six Carryover Blank samples with mixed matrix, and the sampling sequence was Carryover ULOQ, Carryover Blank, Carryover ULOQ, Carryover Blank Cycle the sample six times. In addition, residual effect was changed due to different instruments. Therefore, the residue of each matrix analysis batch was investigated. Injected and analyzed the double-blank samples after the highest detectable concentration standard curve. The maximum peak area response value substance and the internal standard in the double blank sample was not exceeded 20.0% and 5.0% (for the internal standard) minimum peak area response value. We adjusted the sampling sequence method validation sample, and insert additional appropriate double-blank samples. During the analysis biological samples, the impact residues on the determination unknown samples was evaluated, and double-blank samples were inserted between high-concentration samples and low-concentration samples.

4.2 Dilution reliability

Dilution reliability was verified by a dilution factor. The analysis results diluted quality control samples were not used to determine whether the analysis batch meets the accepted standard. The concentration and dilution factor and the diluted QC sample DQC were adjusted according to the actual situation.

4.3 Recovery rate

The measured concentration internal standard was the concentration working solution in the analytical method. After treatment, we added the solution containing the substance under-tested and the internal standard into the double blank sample to make its final concentration consistent with the injection concentration high, medium, and low-quality control samples. The precision recoveries for each concentration level and all concentration levels were ≤ 15.0%.

4.4 Analysis of Batch Capacity

We evaluated the change instrument status with time during sample injection analysis, it was recommended that the accuracy quality control samples used as an indicator was evaluated the sample capacity analysis batch when reviewing the intra- batch accuracy. The number on double-blank samples in the analysis batch was appropriately adjusted to reach the capacity analysis batch.

4.5 System Applicability and analysis batch sampling sequence

Based on the characteristics chromatographic analysis itself, a system suitability test was conducted before formal analysis batch injection to evaluated the instrument system. Before the injection each analytical batch (based on the first sample of the analytical batch, except for the case where the continuous injection does not exceed 24 hours), the sample (matrix sample or solution sample) with a concentration LLOQ, contains both substances or analytical batches containing only solution samples, the solution sample with the lowest concentration. The sampling sequence each analytical batch was double blank sample, zero-point sample, standard curve sample, double blank sample, quality control sample, method validation sample (such as sample for investigating recovery rate and matrix effect), quality control sample, standard curve sample, and double blank sample. The validation samples were dispersed in each set analytical batch quality control samples. The number and injection sequence double-blank samples in the analysis batch was adjusted appropriately.

4.6 Durability

It was recommended to perform partial validation (precision and accuracy single batch) on another instrument with the same configuration to investigate the durability analytical method on different instruments and equipment. When the analysis batch accuracy was pretreated by different lab mates, it was considered that the durability analysis method among different lab mates was investigated at the same time. The durability and the analytical method for pretreatment on different instruments and equipment, by different lab mates and by chromatographic columns with the same model and different serial numbers. Each concentration level QC sample was ≤ 15.0% (except LLOQ QC, the precision was ≤ 20.0%). The deviation between quality control samples accounting for at least two-thirds number and their theoretical values did not exceed ± 15.0% (except for LLOQ QC, the deviation was not exceeded ± 20.0%).

5. STABILITY INSPECTION

The stability inspection was reflecting the environment biological samples or solutions in daily analysis. The stability inspection was adjusted according to the test needs. The stability sample was excluded from the final calculation, such as chromatography or system failure, or problems that occurred during sample preparation or pretreatment and had been recorded in detail. Any data excluded from the calculation was explained.

5.1 Stability of the working solution of the object was tested

The stability working solution substance was tested and investigated at two concentration levels the lowest concentration and the highest concentration working solution. The stability working solution substance was tested at room temperature. After checked it was placed at room temperature for 12 hours. The long-term stability working solution object to be tested was inspected after it was placed at - 20 ° C for 1 week. If the precision exceeds the accepted standard, it was considered that the test result failed due to the instrument, and the stability inspection was repeated without a confirmatory test. If the working solution was found unstable, shorten the storage time of the stability sample or take other measures and re-inspect.

5.2 Stability of internal standard solution

Because the internal standard stable isotope labeling was difficult to obtain. In this case, the stored internal standard solution to the analyte was investigated. The internal standard stock solution was diluted to its working solution concentration. it was considered that the internal standard solution was used and had no impact on the detection. We proved that the stored internal standard solution did not interfere with the determination substance that was tested, this investigation was carried out at least in two analytical batches at the beginning and used internal standard working solution. If no interference was found, the stability internal standard solution was verified.

5.3 Stability of EVT201 and its metabolites Ro 46-1927 and Ro 18-5528 in human plasma at room temperature

The stability EVT201 and its metabolites Ro 46-1927 and Ro 18-5528 in human plasma at room temperature was investigated at two concentration levels the quality control sample with a low concentration (LQC) and the quality control sample with a high concentration (HQC). Each concentration level was three replicates. After placement, we analyzed the stability samples and evaluated their precision and accuracy. If the sample was found unstable, the storage time stable sample was shortened and the investigation was conducted again. The precision on stability samples at each concentration level was ≤ 15.0% and the deviation between the measured concentration at least two-thirds stability samples and their theoretical concentration was not exceeded ± 15.0%. If the precision exceeds the accepted standard, it was considered that the test result failed due to the analysis, and the stability inspection was repeated without the need for a confirmation test.

5.4 Freeze-thaw cycle stability of EVT201 and its metabolites Ro 46-1927, Ro 18-5528 in human plasma at - 20 ° C and - 70 ° C

The stability during the freeze-thaw cycle EVT201 and its metabolites Ro 46-1927 and Ro 18-5528 in human plasma at - 20 ° C and - 70 ° C was investigated at two concentration levels the quality control sample with a low concentration (LQC) and the quality control sample with a high concentration (HQC). Each concentration was repeated at least three times. Between the subsequent freeze-thaw cycles, the stored freezing time was 12 hours apart. The deviation between the measured concentration at least two-thirds stability samples and their theoretical concentration was not exceeded ± 15.0%. and the precision exceeds the accepted standard, it was considered that the test result failed due to the analysis, and the stability inspection was repeated without the need for a confirmation test.

5.5 Long-term stability of EVT201 and its metabolites Ro 46-1927 and Ro 18-5528 in human plasma at - 20 ° C and - 70 ° C

The long-term stability EVT201 and its metabolites Ro 46-1927 and Ro 18-5528 in human plasma at - 20 ° C and 70 ° C was investigated at two concentration levels the quality control sample with a low concentration (LQC) and the quality control sample with a high concentration (HQC). Each density was at least three replicates. The stability samples were tested after being placed at - 20 ° C and - 70 ° C several times (T0, 1 month, 2 months, or other times). After placement, we analyzed the stability samples and evaluated their precision and accuracy. The investigation time was adjusted according to the actual storage time. The precision stability samples at each concentration level were ≤ 15.0%. And the deviation between the measured concentration at least two-thirds stability samples and their theoretical concentration was not exceeded ± 15.0%.

5.6 Stability of EVT201 and its metabolites Ro 46-1927, Ro 18-5528 in human whole blood at room temperature

We had taken three individuals' whole blood from different sources as the blank matrix. After preparation, immediately centrifuged (1700 g, 4 □ C, 10 min) was processed a set sample as the zero-point sample. We compared the ratio average response value at each time point with the zero-point sample. The precision stability sample at each concentration level and each time point was ≤ 20.0%. it was found that the object tested was unstable, the test was repeated, such as adjusting the inspection time (shortening the time), etc.

5.7 Injection reproducibility

The analysis batch for accuracy and precision inspection was re-injected for analysis after being stored at 8 ° C of the automatic sampler for 4 days. The analysis batch samples at least included double-blank samples, zero-point samples, standard curve samples, and quality control samples. The analytical was quantified by the standard curve re-injection, and the results each injection were confirmed to the accepted

standards selectivity, standard curve samples, and quality control samples, we proved that re-injection would not affect the analysis results.

5.8 Stability of treated samples

After an analysis batch has successfully injected samples, its quality control samples (The quality control sample with a low concentration and the quality control samples with a high concentration, at least three replicates for each concentration) were stored at 8 ° C in the automatic sampler for 4 days or a more reasonable period. And then re-injected for analysis. During injection, the freshly prepared standard curve samples were used for quantitative analysis to investigate the stability processed samples placed in the 8 ° C automatic sampler. The precision stability samples at each concentration level were ≤ 15.0%, and the deviation between the measured concentration at least two-thirds stability samples and their theoretical concentration was not exceeded ± 15.0%. If the precision exceeds the accepted standard, it was considered that the test result failed due to the analysis, and the stability inspection could be repeated without the need for a confirmation test.

6.1 ANALYSIS RECORDS

The special record book paper was used to record the test process and data in a timely and standardized manner to ensure that the test records were complete, accurate, and clear. When the record needs to be modified, the original record was kept clear and legible, with the reason for modification indicated, and the date indicated. When data was generated, recorded, processed, stored, and modified in the electronic documents, a validated computer system was used; Record all operations and test personnel and time, Ensure the authenticity, reliability, and traceability data.

6.2 Operating Instructions

Unless otherwise specified, the validation method was strictly referred to SOP-LAB 005 "Validation of Quantitative Analysis Methods for Biological Samples" and SOP-LAB 006

RESULTS & DISCUSSIONS

The method was specific to the detection EVT201, Ro 46-1927, and Ro 18-5528 in human plasma. EVT201 Mean Plasma Concentration Time chart showed the average plasma concentrations on EVT201, a drug, over time in healthy individuals given a single oral dose EVT201 capsules. Figure 1 was presented in the form a graph, with the x-axis showing the time in hours after administration and the y-axis showing the plasma concentration in micrograms per milliliter.

The graph showed that the plasma concentration EVT201 increased rapidly after administration, peaking at around 2 hours, and then gradually decreased over time. Based on the data presented in this chart, we could conclude that the EVT201 capsule was rapidly absorbed by the body and reached its maximum concentration in the blood within a few hours administration. The plasma concentration then gradually decreases over time, indicating that the drug was eliminated from the body through various metabolic processes. These findings were important for understanding the pharmacokinetics EVT201 and for determining the appropriate dosing regimen for this drug. Figure 2 showed multiple oral administrations EVT201 capsules given to the subjects, and the study measures the concentration drug in their bloodstream over time. the mean plasma concentration-time (Mean SD) (PKCS) healthy subjects after multiple oral administrations EVT201 capsule refers to the average concentration drug in the bloodstream over time, as well as the standard deviation average, as measured by pharmacokinetic studies. The term Ro46-1927" likely refers to a specific drug compound, while "Mean Plasma Concentration Time" refers to the average amount drug present in a person's bloodstream over time.

Figure 4 showed typically measured by taking blood samples at various time points after the drug was administered and analyzing the drug concentration in each sample. Multiple oral administrations EVT201 capsules were given to the subjects, and the study measures the drug concentration in their bloodstream over time. The results in this study could help to determine the optimal dosing regimen for EVT201 in future clinical trials and may also inform clinical practice if the drug was approved for use. In summary, the mean plasma concentration-time (Mean SD) (PKCS) of healthy subjects after multiple oral administrations EVT201 capsule refers to the average drug concentration in the bloodstream over time, as well as the standard deviation average, as measured by pharmacokinetic studies.

Analytical method validation is an essential step in pharmaceutical development to ensure that the analytical method used to measure the drug concentration or its metabolites in human plasma was accurate, precise, specific, and reproducible [8]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become the method choice for the determination drugs and their metabolites in biological matrices due to its high selectivity, sensitivity, and specificity [9].

The LC-MS/MS method was tested to ensure that the analytes interest could be detected and quantified in the presence other endogenous compounds in human plasma. A calibration curve was generated by analyzing different concentrations interest in human plasma, and a linear relationship between the peak area and concentration was established [10].

The stability analytes interest in human plasma was assessed under different conditions such as freeze-thaw, long-term storage, and sample preparation. The validation analytical methods for the determination drugs and their metabolites in human plasma by LC-MS/MS showed that the steps mentioned above are common to most validation schemes. However, the specific conditions and criteria for each step may differ depending on the analyte interest, the analytical instrument, and the intended used method.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a powerful analytical technique used for the identification and quantification molecules in a variety biological sample, including human plasma. Chromatographic separation was another important step in LC-MS/MS analysis human plasma, as it allows the separation target analytes from other components sample.

Analytical method validation was a critical process that ensures the reliability, accuracy, and reproducibility an analytical method. The specificity of the LC-MS/MS method pertains to its capability to accurately identify and quantify the target analytes while minimizing interference from other components in the sample [11]. The methods specificity was assessed by analyzing blank plasma samples and comparing them with the spiked plasma samples containing the target analytes [12].

This study showed that the peak time Tmax of EVT201 and the main active metabolite Ro46-1927 after the meal was delayed, the peak concentration Cmax decreased, and the integral AUC plasma concentration-time profile did not change. That was, food affects the drug absorption rate, and food causes the drug absorption rate to slow down and the drug peak concentration to drop; however, it does not affect the extent drug absorption, and the quantity drug absorption remains unchanged [13].

Overall, the validation LC-MS/MS method for the determination Evt201 and its metabolites Ro 46-1927 and Ro 18-5528 in human plasma requires careful consideration various parameters to investigate the accuracy, precision, and consistency method [14]. The validated method can be utilized for the quantifying these analytes in human plasma samples, which has important applications in pharmacokinetic studies, clinical trials, and drug development [15].

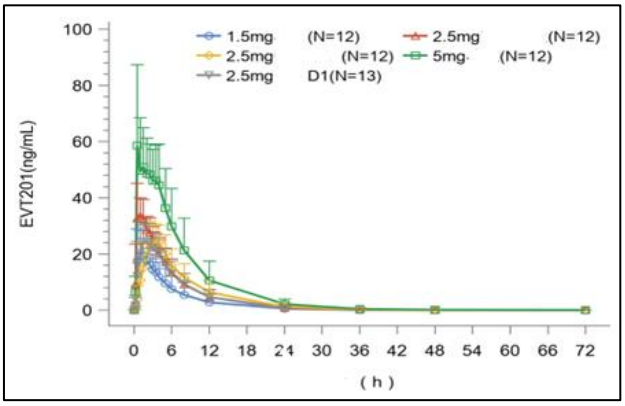


Figure 1: Summary Chart of EVT201 Mean Plasma Concentration Time (Mean SD) (PKCS) of Healthy Subjects after Single Oral Administration of EVT201 Capsule

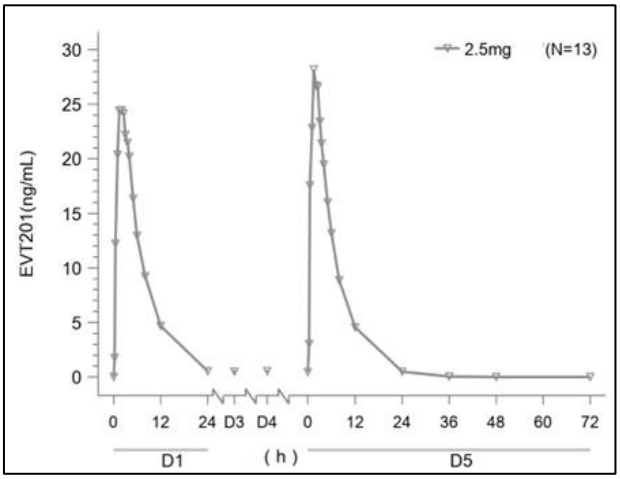


Figure 2: Summary Chart of EVT201 Mean Plasma Concentration Time (Mean SD) (PKCS) of Healthy Subjects after Multiple Oral Administration of EVT201 Capsule

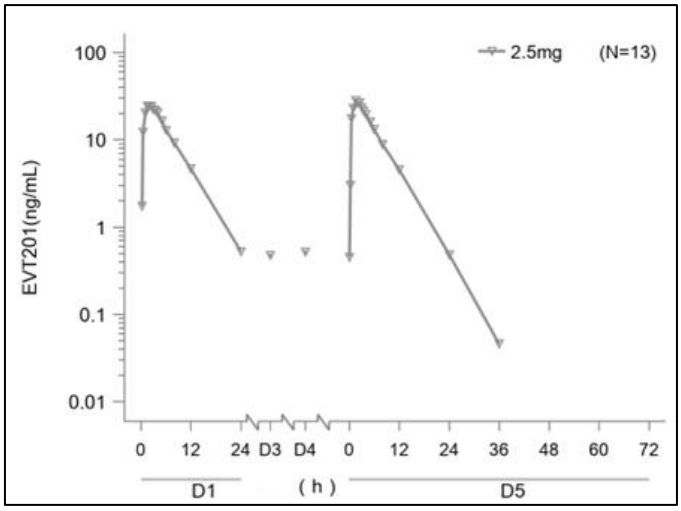


Figure 3: Semilogarithmic Summary Chart of EVT201 Mean Plasma Concentration Time (Mean SD) (PKCS) after Multiple Oral Administration of EVT201 Capsule by Healthy Subjects

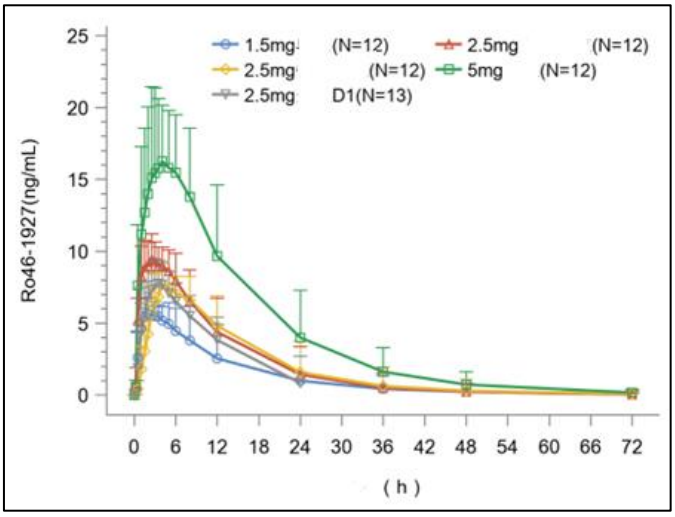


Figure 4: Summary Chart of Ro46-1927 Mean Plasma Concentration Time (Mean SD) (PKCS) of Healthy Subjects after Single Oral Administration of EVT201 Capsule

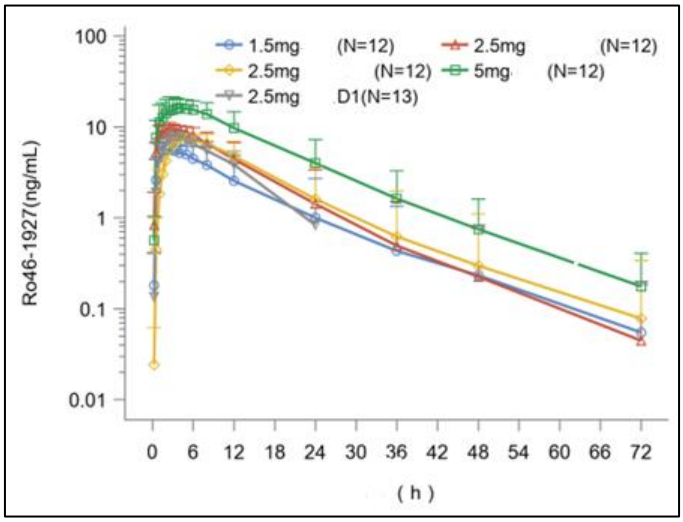


Figure 5: Semilogarithmic Summary Chart of Ro46-1927 Mean Plasma Concentration Time (Mean SD) (PKCS) of Healthy Subjects after Single Oral Administration of EVT201 Capsule

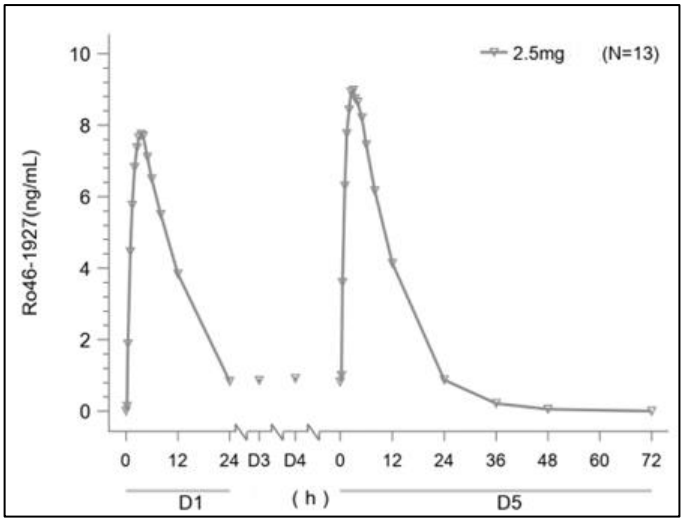


Figure 6: Summary Chart of Ro46-1927 Mean Plasma Concentration Time (Mean SD) (PKCS) of Healthy Subjects after Multiple Oral Administration of EVT201 Capsule

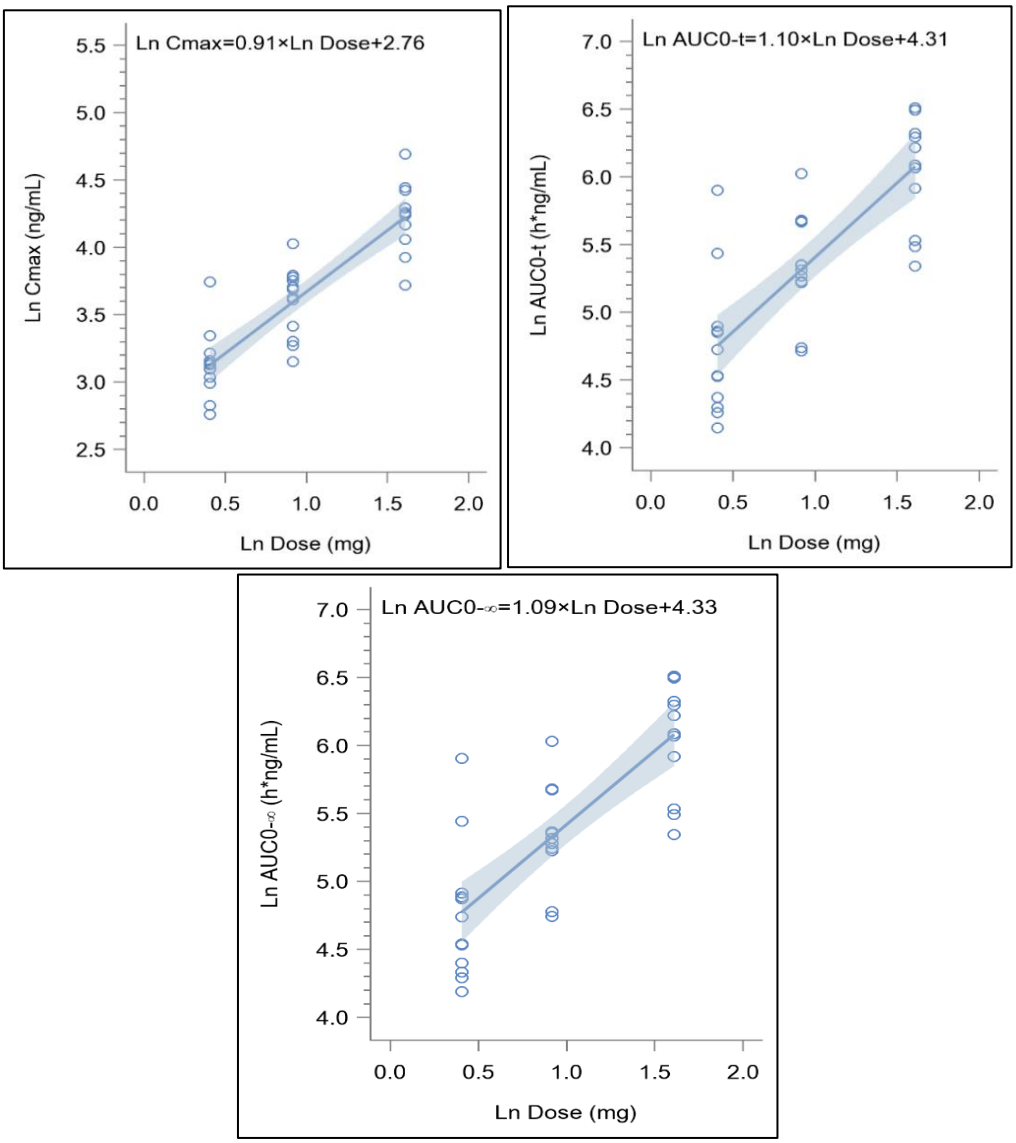


Figure 7: Relationship Diagram (PKPS) of EVT201 Blood Drug Cmax, AUC0-t, AUC0 - ∞ and Dose after Single Oral Administration of EVT201 Capsule to Healthy Subjects

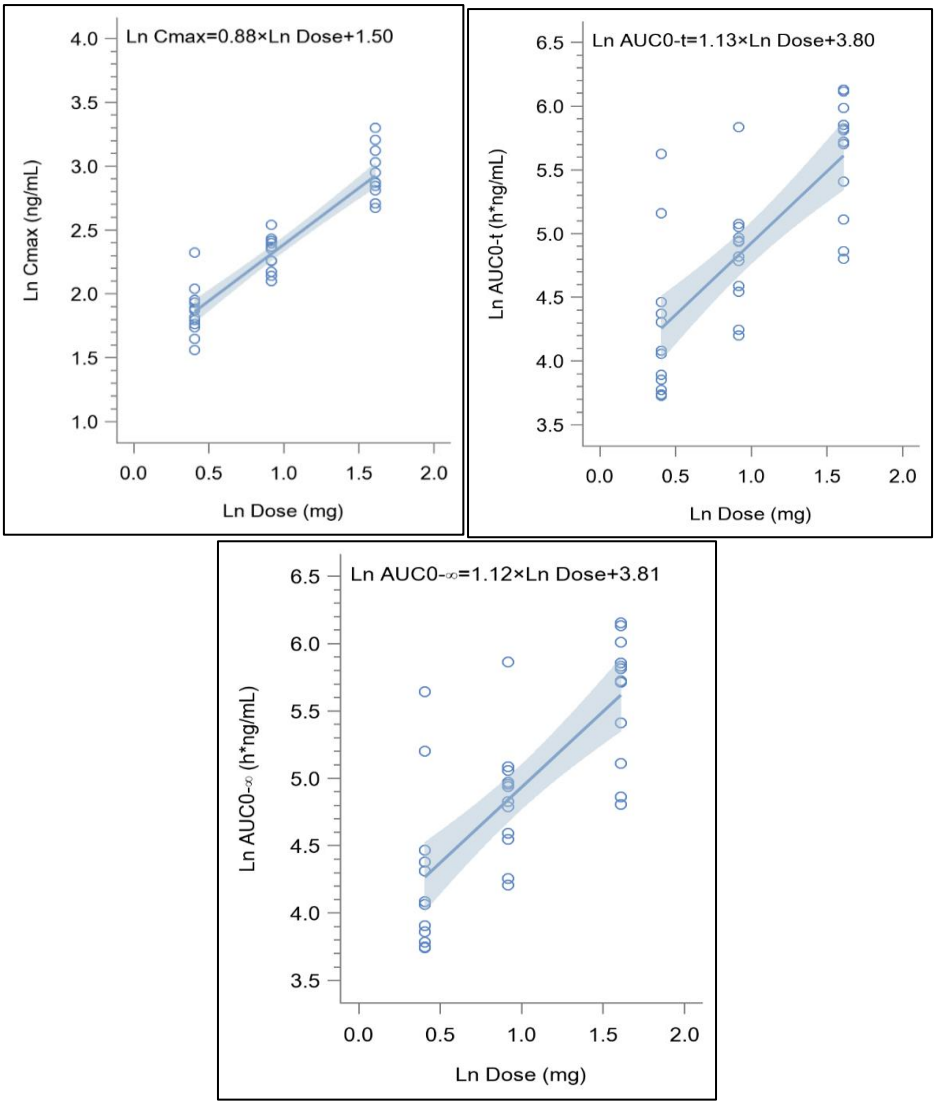
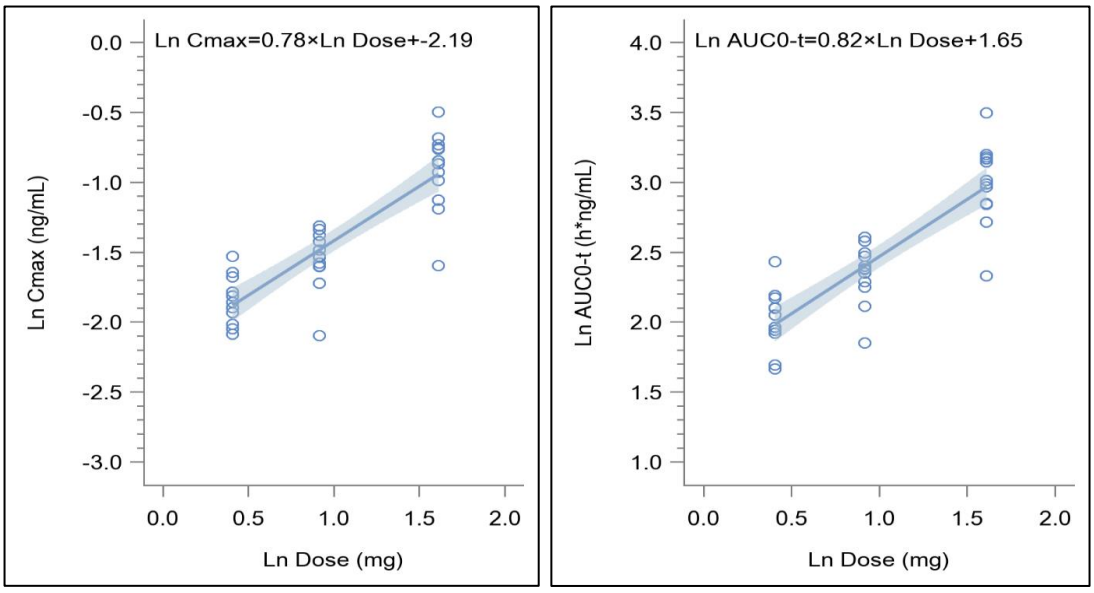


Figure 8: Relationship between Cmax, AUC0-t, AUC0 - ∞ and Dose (PKPS) of Ro46-1927 Blood Drug in Healthy Subjects after Single Oral Administration of EVT201 Capsule on an Empty stomach



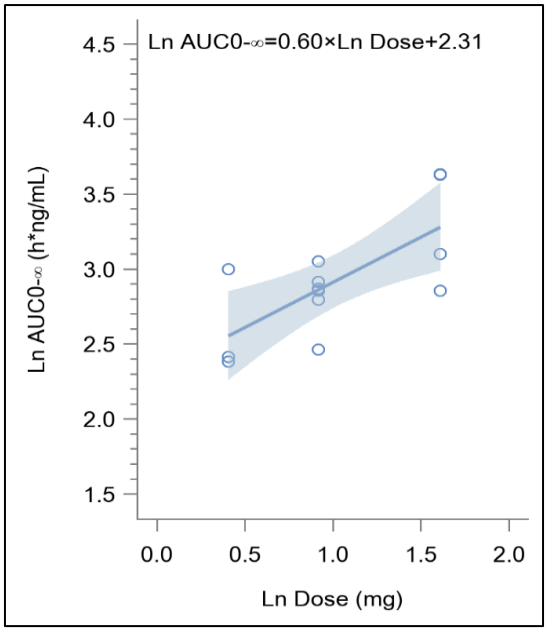


Figure 9: Relationship Diagram (PKPS) of Cmax, AUC0-t, AUC0 - ∞ and Dose of Ro18-5528 in Blood of Healthy Subjects after Single Oral Administration of EVT201 Capsule on an Empty Stomach

Table 1: Relationship between Cmax, AUC0-t, AUC0 - ∞ and dose of EVT201 in the blood of healthy subjects after a single oral administration of EVT201 capsule on an empty stomach

Dose range parameters	Estimated value	SE	95%CI
1.5mg~5mg			
C_{max}(N=36)			
A	2.758	0.092	2.571～2.945
B	0.912	0.084	0.741～1.083
AUC_{0-t}(N=36)			
A	4.308	0.160	3.983～4.633
B	1.099	0.146	0.802～1.396
$AUC_{0-\infty}$(N=36)			
A	4.334	0.158	4.013～4.654
B	1.085	0.144	0.792～1.378

Table 2: Relationship between Cmax, AUC0-t, AUC0 - ∞ and Dose of Ro46-1927 Blood Drug in Healthy Subjects after Single Oral Administration of EVT201 Capsule on an Empty Stomach

	Estimated value	SE	95%CI
1.5mg~5mg			
C_{max}(N=36)			
A	1.504	0.066	1.371～1.637
B	0.883	0.060	0.762～1.005
AUC_{0-t}(N=36)			
A	3.799	0.182	3.429～4.170
B	1.125	0.167	0.787～1.464
$AUC_{0-\infty}$(N=36)			
A	3.811	0.184	3.437～4.186
B	1.123	0.168	0.781～1.465

Table 3: Relationship between Cmax, AUC0-t, AUC0 - ∞ and Dose of Ro18-5528 Blood Drug in Healthy Subjects after Single Oral Administration of EVT201 Capsule on an Empty Stomach

Dose range parameters	Estimated value	SE	95%CI
1.5mg~5mg			
C_{max}(N=36)			
A	-2.193	0.085	-2.366～-2.021
B	0.777	0.078	0.619～0.934
AUC_{0-t}(N=36)			
A	1.653	0.089	1.472～1.833
B	0.817	0.081	0.652～0.983
$AUC_{0-\infty}$(N=36)			
A	2.312	0.197	1.877～2.746
B	0.601	0.179	0.208～0.994

CONCLUSION

A robust LC-MS/MS method was successfully developed and validated for the precise and specific evaluation EVT201 and its two metabolites in human plasma. This validated LC-MS/MS method provides an accurate, precise, and highly specific means for quantifying the presence EVT201 and its metabolites in human plasma. Its robustness and sensitivity make it suitable for a wide range of applications, such as pharmacokinetic studies and bioequivalence assessments, contributing to a deeper understanding the compound's behavior in the human body. The combined precision and accuracy method contribute to its reliability and robustness, enabling precise and reliable evaluation EVT201, and the metabolites present in human plasma samples were analyzed across a wide range of concentrations.

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Declarations of Conflict of Interest

The authors declare no conflict of interest.

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