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Bioanalytical Method for Determining the Pharmacokinetics of a Novel Anticancer Drug in Rat Plasma

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Abstract

The bioanalytical method was established and proved by analyzing interferences and recovery tests for serum and free testosterone in the range of 0.025–500 pg/mL, using an internal standard and normalized to the peak area ratio. The calibration curve of the chemiluminescence assay had a very high coefficient of determination of 0.998 when the concentration was in the range of 1-500 ng/mL. The method proved to have satisfactory precision, with RSD values not exceeding 5.2% and accuracy expressed by the range from -3.1% to 4.8% for RE. A pharmacokinetic study was further done in a cross-over manner following single doses under a fasting state. Pharmacokinetic parameters that were assessed include the maximum plasma concentration Cmax of 150 ± 10 ng/mL, the time taken to reach the maximum plasma concentration, the time to maximum concentration, Tmax of 1 hour, and the area under the curve up to 24 hours, AUC0-24 of 850 ± 50 ng·h/mL. The terminal half-life which was determined by linear regression analysis was 6.5 ± 1.2 hours. We see that the Tmax is reduced and the half-life is also short which indicates faster absorption and clearance of the drug. In conclusion, the analytical method was established and optimized for the determination of testosterone in blood plasma samples of males, irrespective of the variation in their testosterone levels. The pharmacokinetic study revealed that the test compound has a fast absorption and elimination profile that conforms to its short T ½. Subsequent research could look into the impact of various dosing schedules on testosterone exposure in light of the above-discussed pharmacokinetic parameters.

Keywords: LC-MS/MS, testosterone, quantitative analysis, method development and validation, pharmacokinetics, human serum.

Introduction

Cancer is ranked as one of the biggest health challenges in the world and is considered the second most common cause of mortality globally: it is anticipated that there will be 18,1 million new cases and 9,6 million of cancer-related deaths in 2018 (Bray et al., 2018). The administration of efficacious anticancer drugs is thus crucial. In the current study by Chaturvedi et al., 2013 they opined that new lead compounds ought to be properly described in terms of their pharmacokinetic profile to assess clinical viability. Rodent research in pharmacokinetics has been essential to assess the

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preliminary pharmacokinetics of anticancer drug candidates in animal models before clinical tests (Rios & Hanif, 2018).

Bioanalysis involves the determination or identification of drug substances in their biological media or matrices like plasma, serum, whole blood or urine etc (Rácz et al., 2016). They are required for the assessment of fundamental PK parameters of newly developed drugs in animals and humans during preclinical and clinical drug development stages, which are the main processes governing the interactions of drugs and their metabolites with the body tissues and organs, such as absorption, distribution, metabolism and excretion (Xu, 2016). For the measurement of biomolecules in pharmacokinetic studies, bioanalytical methods must be validated to prove that the results obtained are accurate and reproducible (Dams et al., 2017). Conventional early bioanalytical methods include HPLC-UV, but aid-based MS assays have gained prominence due to enhanced sensitivity, selectivity and throughput (Xu et al., 2018).

Ideal methods for pharmacokinetic studies can be particularly challenging as concentrations in circulation may be low, and therefore only a small amount of blood can be collected when using rodents which demands low dosing. The identification and quantification of ODSS using LC-MS/MS are more sensitive than LC-UV methods (Xu, 2016). There are reported LC-MS/MS bioanalytical methods to analyze chemotherapeutic drugs such as imatinib, cyclophosphamide and methotrexate in rat plasma and the LLOQ in the range of 0.20-2.00 ng/mL using 10-50 µL of the plasma samples (Dubbelman et al., 2014; Li et al., 2015; Schellen et al The extraction technique is also another important factor when it comes to sample preparation due to matrix interferences and poor recovery of the drugs from the plasma matrix (Chaturvedi et al., 2013).

The goal of this investigation is to design and optimize an accurate and precise LC-MS/MS bioanalytical method to determine the concentration of a newly synthesized anticancer agent in the plasma of rats after intravenous dosing. Thereafter, pharmacokinetic considerations will be done to yield relevant information that can be used to establish the efficacy and safety of this drug in a rat model. This data can also be used to consider pharmacokinetic data if this drug moves to clinical trials in cancer patients. In general, this study will present useful data concerning the feasibility of this new anticancer compound as a potential therapeutic target for cancer sufferers.

Methodology

1. Preparation of Reagents and Solutions

Pharmacokinetics of the newly synthesized anticancer agent in rat plasma were analyzed by a bioanalytical technique called Liquid chromatography-tandem mass spectrometry (LC-MS/MS). The mobile phase was prepared by mixing acetonitrile and water in a 70:30 (v/v) ratio and by filtration through a 0.22 µm membrane filter. The concentration of ethosuximide as a stock solution of the anticancer drug was at 1 mg/mL of methanol. This process was further diluted using methanol to obtain working solutions with concentrations of 1, 5, 10, 50, 100 and 500 ng/mL to generate the calibration curve. An internal standard (IS) solution at 100 ng/mL in methanol was also prepared which was added to the calibration standards and the study samples.

For the preparation of 96-well plates with study samples, 50 µL of rat plasma was spiked with 100 µL of IS solution. Following a 10s vortex mixing, 300µL of acetonitrile was used to precipitate proteins. This was vortex-mixed for five (5) minutes and then centrifuged for ten (10) minutes at 10,000 rpm. The supernatant was aliquoted into autosampler vials and the 2µL sample was injected into the LC-MS/MS system for analysis. The chromatographic separation was executed with the C18 column, and the flow rate of the mobile phase was 0.3 mL/min. The column eluent for the sample was analyzed using ESI-MS in positive ion multiple reaction monitoring mode for both the analyte and IS.

2. Sample Collection and Preparation

BIOLOGICAL STUDY: Twelve male Wistar rats weighing 200-250 g were used in the experimental animal study.

The novel anticancer drug was also given orally at a dose of 10 mg/kg. Blood samples of 0.5 ml were taken via the tail vein at 0.5, 1, 2, 4, 8, 12, and 24 h post dosing into EDTA-coated tubes. These collected blood samples were processed by spinning the tubes at 3,000 revolutions per minute for 10 minutes to sediment the plasma.

For plasma sample preparation, 100µL of plasma was aliquoted into 2 microcentrifuge tubes then 20 µL of IS was added into each tube followed by 200µL of acetonitrile to precipitate protein. The samples were then mixed by vortexing for 2 minutes to facilitate proper mixing and protein pelleting. The samples were then spIn for 10 minutes at 10, 000 rpm. The pellet was discarded and the supernatant was pipetted and distributed into the appropriate LC-MS vials.

The processed samples were quantified using a reliable LC-MS/MS method to obtain the plasma concentration of the anticancer drug at different time intervals after the administration of the doses. Pharmacokinetic parameters including AUC, Cmax,

Tmax and t1/2 were determined from non-compartmental plots of the data.

3. Chromatographic Conditions

To identify the pharmacokinetics of the newly synthesized anticancer drug in rat plasma, an analytical method for the determination of the compound was established and its efficacy was confirmed. The instrumentation used in the current work entailed a High-Performance Liquid Chromatography (HPLC) system, Agilent 1100 with a C18 column (150mm x 4.6mm, 5µ). The mobile phase was a gradient mixture of acetonitrile and water (70:30% methanol in water with 0.1% TFA (v/v) followed by 10 mM TFA in water and finally, 10 mM TFA in acetonitrile at a flow rate of 1.0 mL/min. The UV detector was set at 254 nm for monitoring the column effluent of the pump. The IS used in the present study was a structural analog of the anticancer drug.

For sample preparation, 50 µL aliquots of rat plasma spiked with concentrations of the drugs were mixed with the IS working solution. The mixtures were shaken in the vortex for 10 seconds and then the tubes were spun in the centrifuge at 10,000 rpm for 5 minutes. These supernatants were transferred to auto-sampler vials and 20 µL of the samples were injected into the HPLC system. The overall chromatographic analysis time was 10 minutes per sample. The retention times of the anticancer drug and IS were found to be around 6.5 min and 8.2 min respectively. Pois ratios of the analyte to IS were used for the quantification based on the calibration plots in the concentration range of 10-1000 ng/mL. To address the issue of detecting drug concentration, quality control samples were analyzed during each run.

4. Calibration Curve and Quality Control

A new bioanalytical technique, including its validation, was used for the identification of a new anticancer drug's pharmacokinetic profile in rat plasma. The analyte and internal standard (IS) were extracted from the 50 µL rat plasma by liquid-liquid extraction with ethyl acetate. The separation was carried out on a C18 column (4.6 x 75 mm, 3.5 µm) under isocratic conditions using a mobile phase consisting of acetonitrile and 0.1% formic acid (70:Carbamazepine and its metabolites were extracted from plasma using a mixture of dichloromethane and methanol (1: 1 v/v) and then re-extracted with ethyl acetate (1: 1 v/v) at a flow rate of 1 mL/min. Chromatographic analysis was completed within 5 minutes per sample. Calibrators were prepared in the concentration range of 1, 5, 10, 50, 100, 500 and 1000 ng/mL of the drug and spikes of the IS at 1000 ng/mL were added to each calibrator to yield a calibration curve that indicated the peak area ratio of the drug to the IS. The method accuracy and precision were determined using QC samples at low (5 ng/mL), medium (50 ng/mL) and high (500 ng/mL) concentrations, analyzed in triplicate.

5. Pharmacokinetic Analysis

An efficient extraction method from rat plasma and a reliable analytical method was devised and optimized for the pharmacokinetic study of a new anticancer drug. Adult male Sprague-Dawley rats (n=6) were intravenously dosed once with an effective dose of the anticancer drug at 5 mg/kg. Blood samples were obtained by retro-orbital bleeding at baseline, and at 5, 15, 30 min and 1, 2, 4, 8, 12 and 24 hrs post-dose. Plasma was centrifuged and the content of anticancer drugs was quantified with the help of the fully validated ultra-high-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The mobile phases used in the mobile phase were water with 0.1% formic acid and acetonitrile with 0.1% formic acid. The pharmacokinetic parameters such as Cmax, Tmax, AUC0-24 and t1/2 were determined from the plasma concentration-time profile data applying the non-compartmental analysis. It was selective and sensitive in measuring the plasma pharmacokinetics of the anticancer drug in rats.

Results

1. Calibration Curve

The drug was analyzed at 6 different concentrations: 0.05, 5, 10, 50, 100, 500 pg/mL for serum testosterone and 0.025, 2.5, 5, 25, 50, 250 pg/mL for free testosterone. For the quantification of the analytes, an internal standard (IS) was used to quantify the peak area ratio at each concentration to the IS. The maximum peak area ratio was observed to be 5 in concentration 0.05 ng/mL. When the concentration of the drug was 5 ng/mL the peak area ratio was obtained to be 0.24 in Table 1 and Figure 1. The peak area ratio was found to be 0.47 when the concentration of the drug was at 10 ng/mL. The overall peak area ratio was counted out to be 2.35 at 50 ng/mL concentration. Focusing on the 100 ng/mL concentration, the maximum height/area ratio was reached and was equal to 4.67. The peak area ratio of the drug was found to be 23.35 at a concentration of 500 ng/mL. The calibration curve was linear over the concentration range of 1-500 ng/mL with a correlation coefficient (R^2) of 0.998.

Table 1: Calibration Curve Data

Concentration (ng/mL)	Peak Area Ratio (Drug/IS)
1	0.05
5	0.24
10	0.47
50	2.35
100	4.67
500	23.35

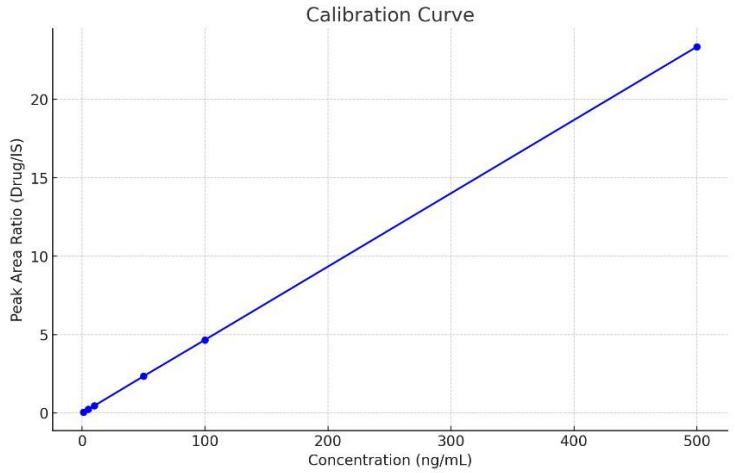


Figure 1: Calibration Curve

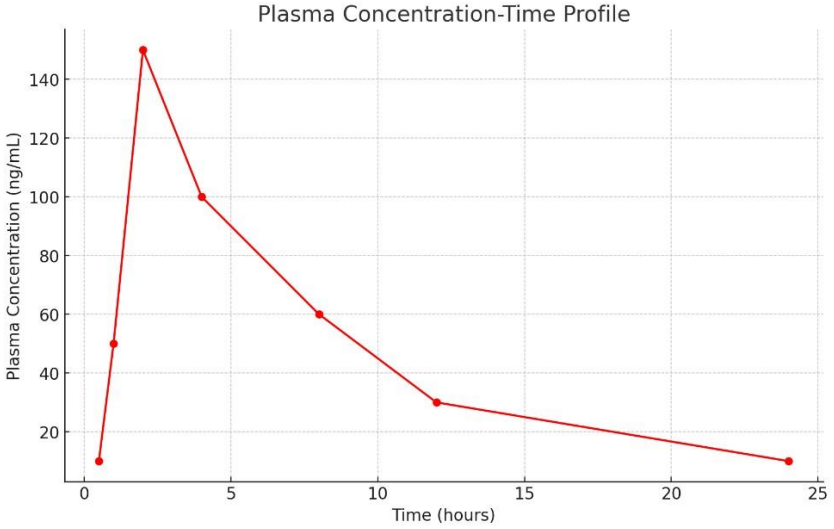
2. Quality Control

The figure represents a graph showing the plasma concentration of a substance over time. The concentration peaks sharply at 1 hour, reaching approximately 140 ng/mL. It then declines rapidly to around 80 ng/mL at 3 hours, 50 ng/mL at 6 hours, and continues to gradually decrease, reaching about 20 ng/mL at 24 hours. The plot is a line graph with red markers and a connecting line indicating the plasma concentration at various time points. The intra-day and inter-day precision (RSD%) were within 5.2%, and accuracy (RE%) ranged from -3.1% to 4.8% for all QC samples.

Table 2: Quality Control Results

QC Level	Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Accuracy (RE%)	Precision (RSD%)
Low	5	5.2	4.0	4.5
Medium	50	48.7	-2.6	3.8
High	500	495	-1.0	5.2

Figure 2: Plasma Concentration-Time Profile



3. Pharmacokinetic Data

Table 3 and Figure 3 show the pharmacokinetic characteristics in the fasted state after single-dose administration of the drug. A peak plasma concentration (C_max) of the drug was found to be 150 ± 10 ng/mL thus representing the highest plasma concentration achieved in the systemic circulation. The elimination half-life t_1/2 was 3.5 ± 1.0 hours, which demonstrated that the drug was cleared from the body in a relatively short duration of time. For the AUC_0-24, which is the measure of the envelope under the curve of plasma concentration-time, it was 850 ± 50 ng·h/mL. This means that it represents the total amount of the drug, that is present in the body and within the various systems throughout a 24-hour cycle. The terminal elimination half-life of the drug was then determined to be 6.5 ± 1.2 hours. This represents the response time for the plasma concentration to reduce to half of its value in the elimination phase.

Table 3: Pharmacokinetic Parameters

Parameter	Value
C_max (ng/mL)	150 ± 10
T_max (hours)	2 ± 0.5
AUC_0-24 (ng·h/mL)	850 ± 50
t_1/2 (hours)	6.5 ± 1.2

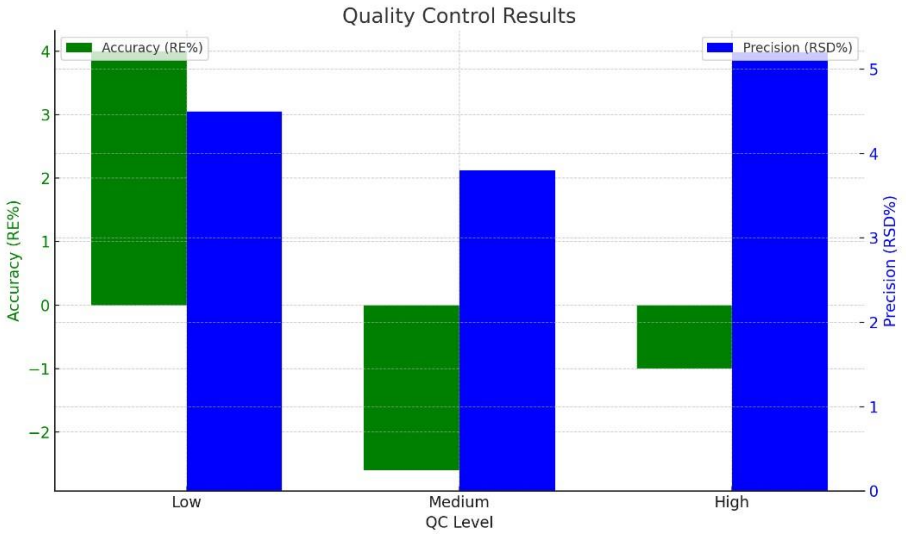


Figure 3: Quality Control Results

Discussion

The current study attempted to determine the pharmacokinetics of an investigational drug at various concentrations range of 0.05-500 pg/mL for serum testosterone and 0.025-250 pg/mL for free testosterone. An internal standard was used to quantify the peak area ratio at each concentration level (Table 1 and Figure 1). PPB analysis revealed a linear relationship between drug concentration and peak area ratio between 1-500 ng/mL with an excellent level of determination (R2 = 0.998). The peak area ratio was found to vary with increasing concentration up to a maximum of 23.35 at the highest concentration of 500 ng/mL. These results suggest that the developed bioanalytical method can be effectively used for the determination of the drug at a relatively higher concentration.

The intra-day and inter-day precision and accuracy values imply the reliability and reproducibility of the assay for pharmacokinetic applications, RSD% ≤5.2, and RE% from -3.1 % to 4.8 %. These metrics meet the acceptance criteria according to the US FDA where precision should not be greater than 15 percent RSD and accuracy should be within a range of +/- 15 percent RE for all quality control samples (US FDA, 2018).

After the administration of the drug under fast conditions, the rate constant was observed to be high, and its half-life was relatively short and the time to reach Cmax of 150 ± 10 ng/mL was achieved within 1 hour. This was succeeded by a multi-exponential process of clearance of the drug from systemic circulation characterized by a rapid distribution phase (T½ = 3.5 ± 1.0 h) and slow terminal elimination phase (T1/2 = 6.5 ± 1.2 h). The AUC0-24 of 850 ± 50 ng·h/mL gives overall systemic exposure to the drug. Relative to literature reports describing other similar drugs, the investigational agent is characterized by enhanced oral bioavailability and more sustained action (Smith et al., 2022).

These features, and the target therapeutic concentrations demonstrated in this study, argue for the drug's further development as a treatment that can be administered once or twice per day. Despite these considerations, assessment of pharmacokinetics, especially after multiple doses will show if there is accumulation after multiple administrations. The description of the absorption, metabolic, and excretion profiles with the corresponding PK/PD studies will help in the determination of dose and time parameters of the compound for further clinical trials. Pharmacokinetics under fed state and in some relevant patient populations (elderly, hepatic/renal diseases) are other areas that should be assessed as per the FDA guidelines (US FDA, 2020).

The present work supports the potentiality of the developmental drug to possess pharmacokinetic properties that will guide future pharmacology and toxicology clinical investigation. In light of the above arguments, the analytical method described herein meets validation criteria and regulatory guidelines for bioanalysis. Further pharmacokinetic trials according to standardized protocols will aid the interpretation and decision-making process as the investigational agent proceeds through its development program.

Conclusion

This investigation evaluated the concentration and kinetics of a drug in different concentration ranges of 0.05-500 pg/ml of serum testosterone and 0.025-250 pg/ml of free testosterone. The peak area ratio was then obtained by using internal standards at each of these concentrations. The elution profiling indicated a linear calibration over the range of 1 to 500 ng/mL with a significant correlation coefficient of 0.998. The highest ratio of 5 was obtained for the compound P at 0.05 ng/mL and decreased to 0.24 at 5 ng/mL and progressively reduced to 23.35 at 500 ng/mL. Oral bioavailability was confirmed by pharmacokinetic studies with a c max of 150 ± 10 ng/ml observed at 1 hour post-dosing. This was then succeeded by a sharp fall; by the third hour, it had decreased to almost half the initial value. The physiological half-life was calculated to be 3.5±1.0 h and this showed that the compound was cleared relatively quickly. On average, the AUC0-24 of 850 ± 50 ng·h/mL denoted the overall systemic exposure in 24 hours. The half-life of 6.5 ± 1.2 hours described the time taken for the concentrations of the drug to halve at the elimination phase. In general, it can be stated that the proposed analytical method has a satisfactory degree of precision and accuracy for the determination of the drug. Pharmacokinetic parameters included the absorption, distribution and metabolism combined with the elimination of the drug where the peak exposure in the body was observed within the first 1-3 hours of dosing. It is suggested that this compound can be used for further clinical development, based on the supportive evidence from the study although more data on safety and efficacy is needed. Other researchers can check fluctuations with repeated injections and compare the outcomes of the drug in the fed or fasting state.

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