# RESEARCH METHODOLOGY
# LEC. 3

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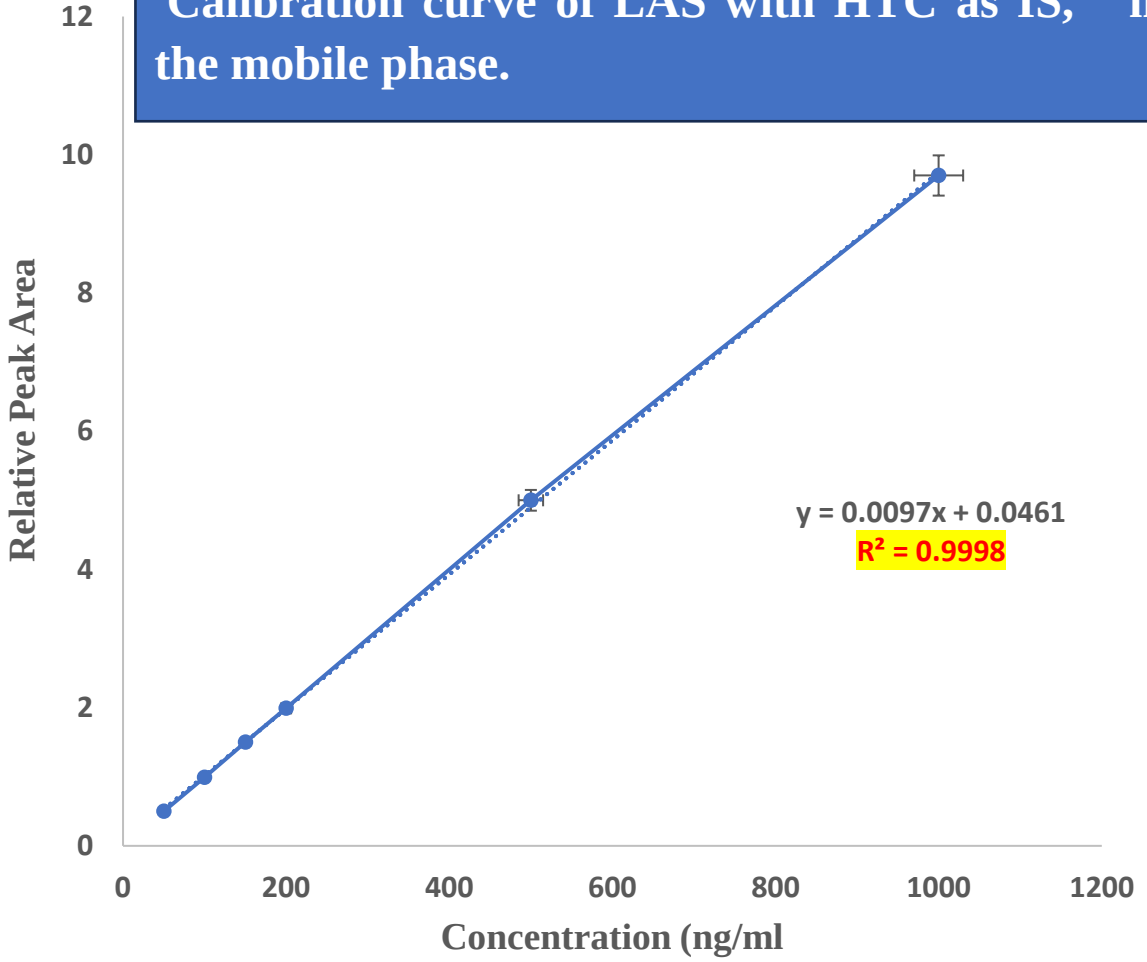
# Method of Validation of HPLC

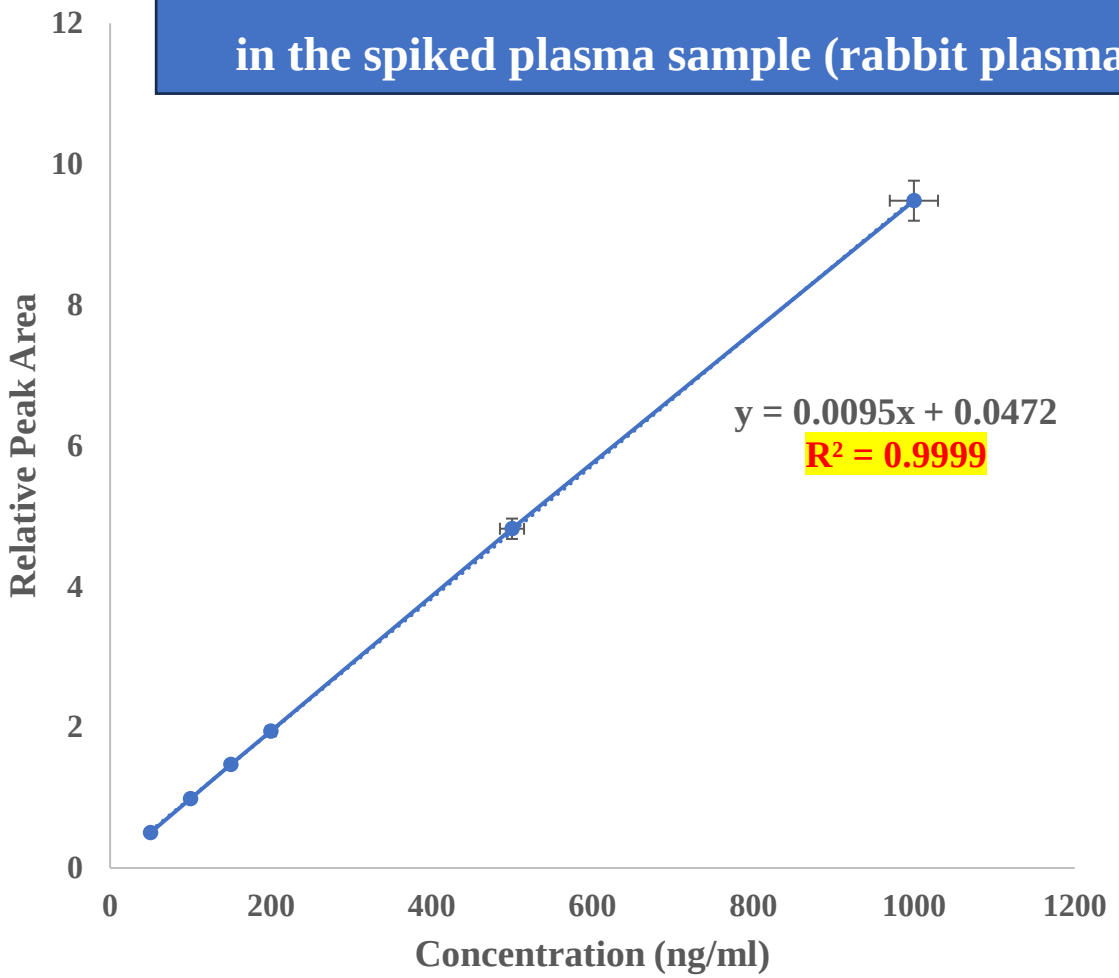
## 1. Linearity

- The linearity of the proposed method was verified by constructing calibration curves at various concentrations (ranging from **50 to 1000 ng/ml**) of drug (LAS) using hydrochlorothiazide (HTC) as the internal standard (IS) at a concentration of **50 ng/ml**.
- Using a linear least squares regression analysis, calibration curves were generated for LAS in the **mobile phase and spiked plasma samples** by plotting their **relative peak area** (**ratio of the peak area of the drug to the peak area of IS**) against their **respective concentrations**.

**Calibration curve of LAS with HTC as IS, in the mobile phase.**




**Calibration curve of LAS with HTC as IS, in the spiked plasma sample (rabbit plasma)**

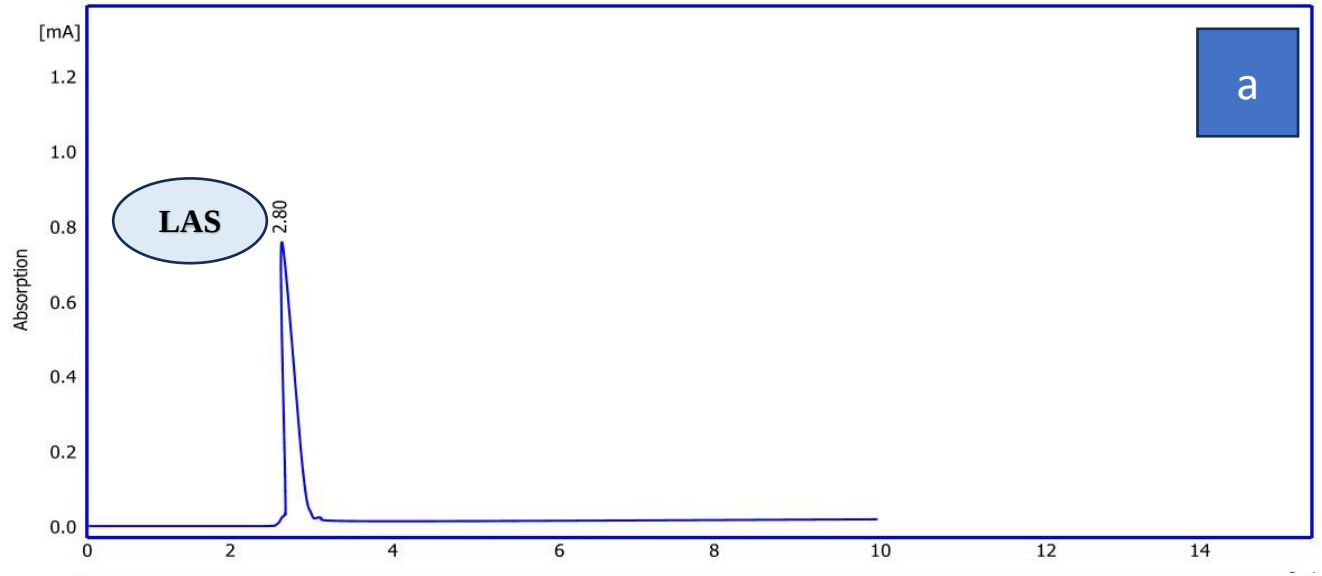


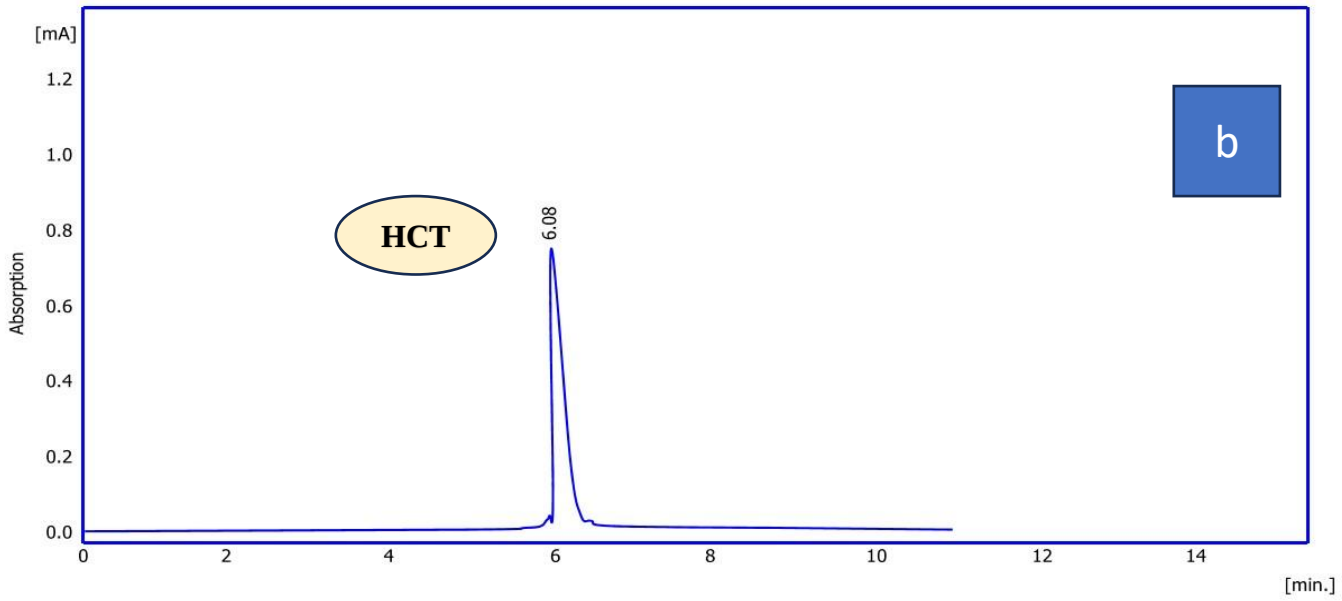

**The RPA yielded a linear correlation over the predicted concentration range for the Calibration curves of LAS which were constructed once in the mobile phase and other in the spiked plasma samples.**

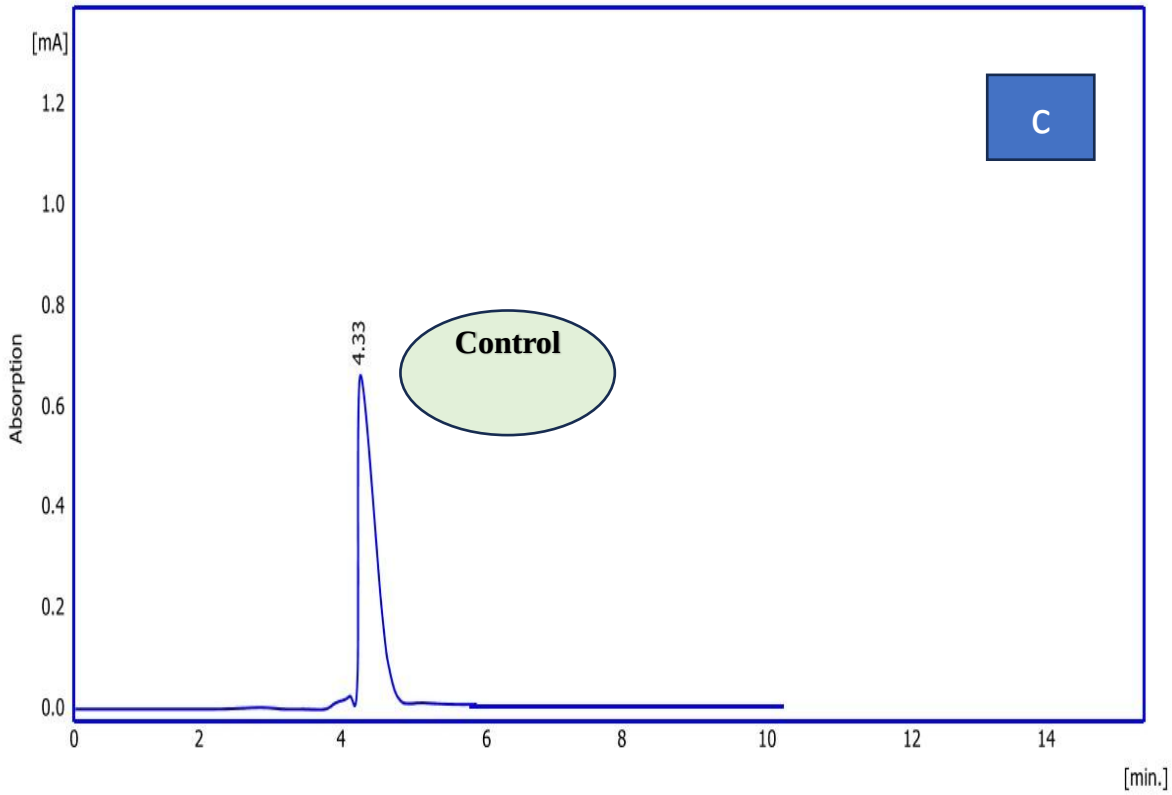
## 2. Specificity/Selectivity

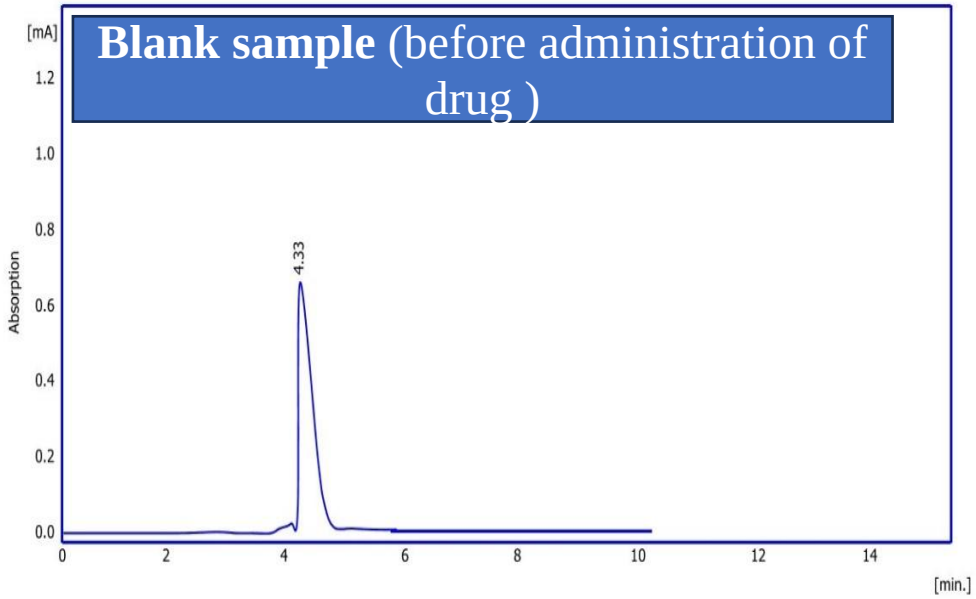
The investigation of the analytical method's specificity and selectivity involved verifying that the required peak area of the **drug** under study and its corresponding **IS** were **completely separated** and resolved in the mobile phase and spiked rabbit plasma samples with suitable concentrations of each compound.

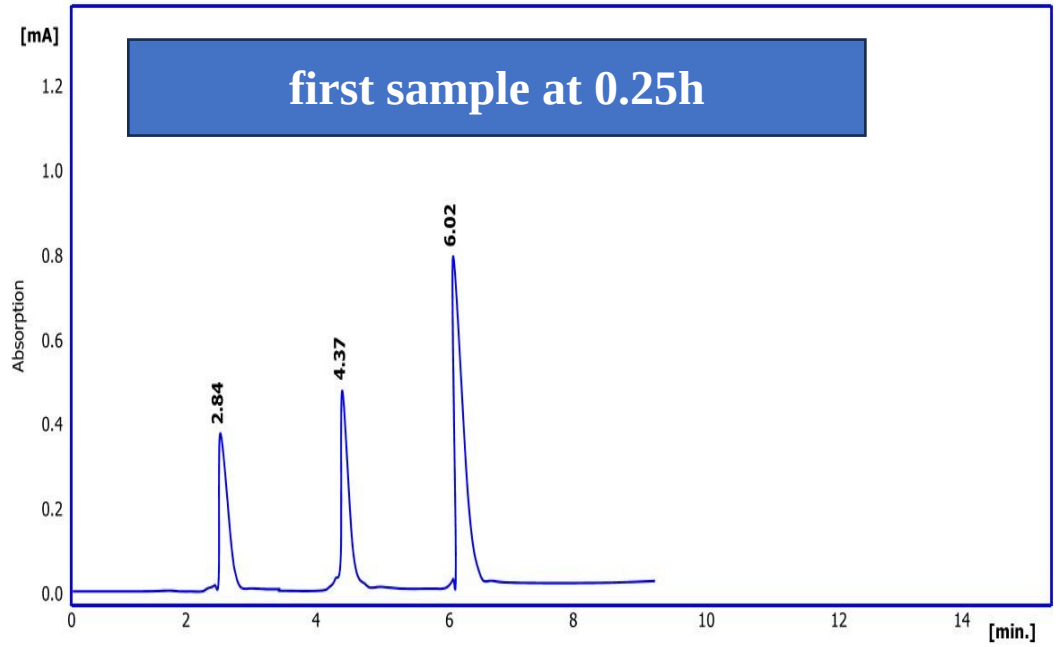
- Figures (a, b & c) show the retention time and peak chromatograms of LAS (standard solution), hydrochlorothiazide (internal standard solution), and plasma sample(control) to be equal to 2.8, 6.08, and 4.33 min, respectively.

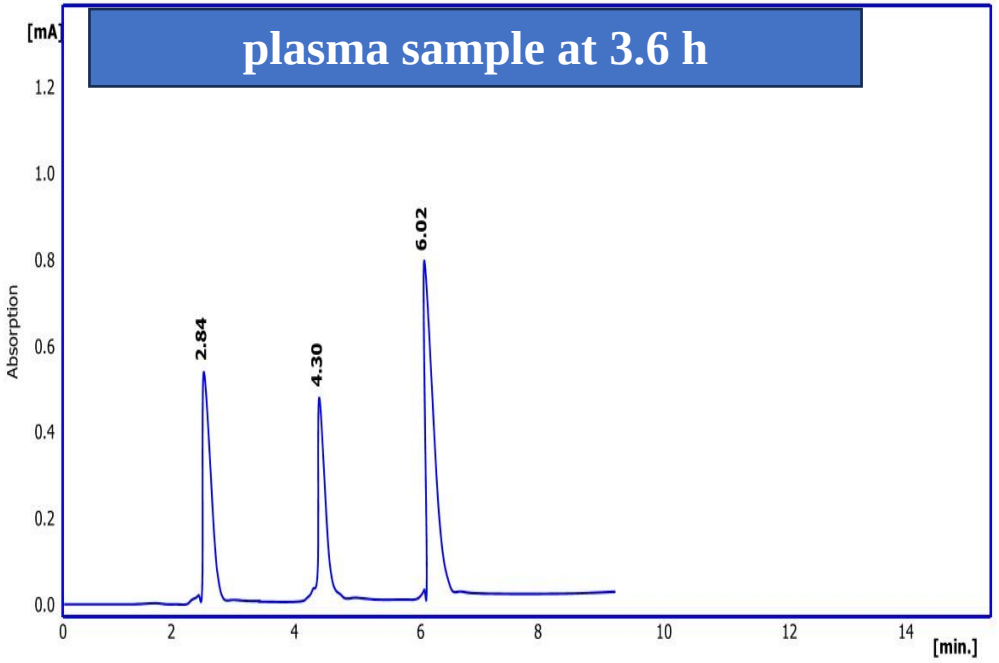


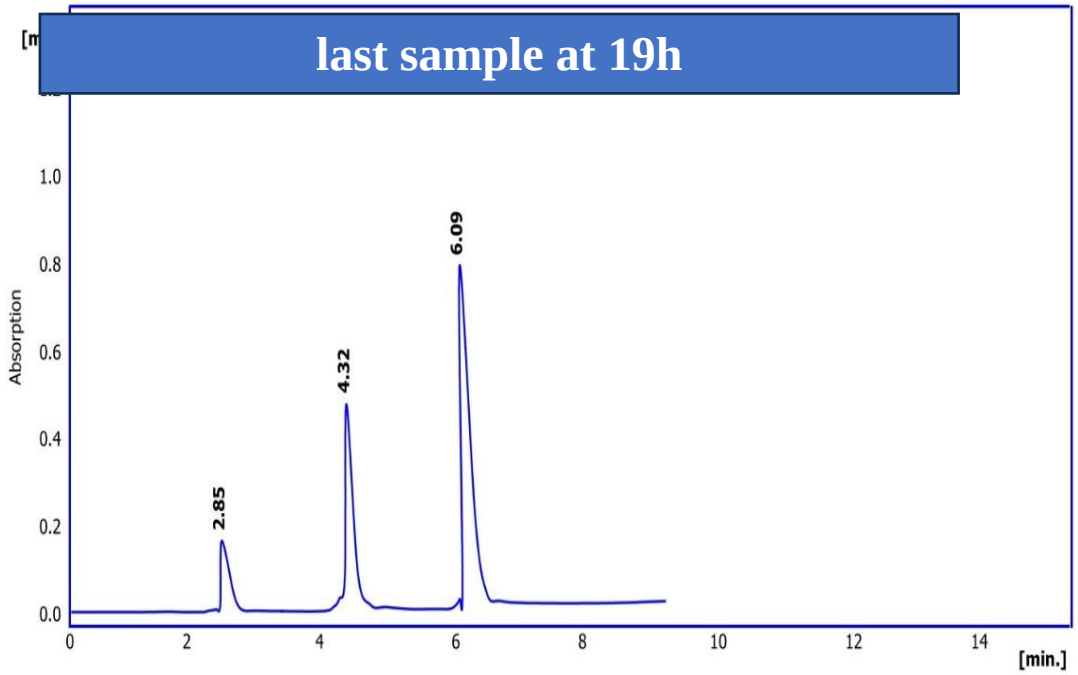




Blank sample (before administration of drug )
[mA]
Absorption
4.33
[min.]


first sample at 0.25h
[mA]
Absorption
2.84
4.37
6.02
[min.]


plasma sample at 3.6 h
[mA]
Absorption
2.84
4.30
6.02
[min.]


last sample at 19h
Absorption
2.85
4.32
6.09
[min.]

- Chromatograms of blank(control) and spiked(test) plasma samples of **drug** at different concentrations with their **respective IS** (HCT) at constant concentration, confirming that:

- The peaks were **completely separated**, and there **was no interaction** or overlap.
- The regression in the **intensity or response** of the drug(LAS) peak while the HCT peak **remains approximately at the same intensity** all over the samples would be in accordance with decreasing the concentration of the LAS with time(elimination phase ), keeping the HCT concentration constant in each spiked sample.

# 3. Quantification (LLOQ)

By injecting a 100 µl sample and employing the dilution method with a signal-to-noise (S/N) approach, the limits of detection and quantification were ascertained. In determining the lower limit of detection (LLOD), a minimum concentration was identified at which the signal-to-noise ratio (S/N≈3) was no less than three. Similarly, the LLOQ was established at the minimum concentration where the signal-to-noise ratio (S/N≈10) was no less than ten.

| intercept 1(c1) | c2 | c3 | sd of c | sd/b | | |
|---|---|---|---|---|---|---|
| 0.001 | 0.0694 | 0.0609 | 0.037280066 | 3.883340226 | 11.7 | **LOD(3x sd/b)** |
| | | | | | 38.8 | **LOQ(10 x sd/b)** |

The Lower limit of detection (LLOD) for drug standard solutions was found to be **11.8** ng/ml, while the lower limit of quantification (LLOQ) was found to be **39.4** ng/ml.

The sensitivity of the analysis method has been suggested with these results, as well as it complies with the last drug sample that could be detected·

## 4. Accuracy

The accuracy was assessed by determining **the percentage recovery (%)** of **injected drug solutions** and blanks (**using a mobile phase** for dilution or dilution solvent) at three concentrations (**40, 80, and 100** ng/ml) while maintaining the **IS concentration constant** (50 ng/ml of HCT) in each sample. In triplicate, the samples were injected into the HPLC system.

The percent recovery for the drug was calculated using the following equation:

**% Recovery = ([A] / [B] *100)**

**Where [A] is the net peak area of the drug in the plasma sample, and**

**[B] is the peak area of the drug in a standard mixture (mobile phase).**

- The accuracy of the proposed analysis method was indicated based on **percent recovery** at three concentration levels, 40, 80, and 100ng/ml for the drug
- The average percent recovery for the drug was found to be 98.37%, as shown in the Table below:
- According to the standard guidelines, the suggested analysis method was considered **accurate** since the drug's excellent extraction efficacy was obtained.

| Concentration(ng/ml) | RPA in spiked plasma | RPA in the mobile phase | %Recovery |
|---|---|---|---|
| 40 | 0.4272 | 0.4341 | 98 |
| 80 | 0.8272 | 0.8461 | 97.8 |
| 100 | 0.998 | 0.996 | 99.3 |
| AVERAGE | | | 98.37 |

## 5. Precision

- The relative standard deviation (RSD) of **repeatability** (injection and analysis) and **inter-day reproducibility** (three separate days (n = 3 × 3) utilizing the same instrument) is used to express precision.

**Intra-day reproducibility** was assessed on the same day (n = 3) using the same instrument.

- The standard solutions, which were prepared in the **mobile phase**, and **spiked plasma** of the drug were each prepared at a concentration of **50 ng/ml** with a corresponding constant concentration of IS (**50 ng/ml of HCT**).
- These solutions were injected into the HPLC system three times daily for three consecutive days.
- The mean value for relative peak areas (RPA) was ±SD, and the %RSD was computed using the acquired data.

# Inter and Intra-day reproducibility.

| IN mobile Phase | day 1-response | day2-response | day 3-response | in spiked | day 1-response | day2-response | day 3-response |
|---|---|---|---|---|---|---|---|
| | 990.08 | 993.55 | 990.14 | | 990 | 993.2 | 989 |
| | 998.14 | 986.08 | 999 | | 998 | 980 | 998 |
| | 996 | 988.89 | 991.04 | | 989.5 | 988.6 | 990 |
| | | | | | | | |
| | day 1-RPA | day2-RPA | day 3-RPA | | day 1-RPA | day2-RPA | day 3-RPA |
| | 0.4988 | 0.5005 | 0.4988 | | 0.498741 | 0.500353 | 0.498237 |
| | 0.5028 | 0.4968 | 0.5033 | | 0.502771 | 0.493703 | 0.498237 |
| | 0.5018 | 0.4982 | 0.4993 | | 0.498489 | 0.498035 | 0.498237 |
| | | | | | | | |
| AVERAGE | 0.5011 | Mean RPA | | | 0.50000 | | |
| SD | 0.002103323 | | | | 0.00240 | | |
| RSD% | 0.419719401 | | | | 0.48057 | | |
| | | | | | | | |
| AVERAGE days | 0.5000 | Mean RPA | | | 0.4985 | | |
| SD | 0.0022 | | | | 0.0024 | | |
| RSD% | 0.4438 | | | | 0.4766 | | |

# repeatability

| **Repeatability** | day -1 | day 1-response |
|---|---|---|
| | 990.08 | 990 |
| | 998.14 | 998 |
| | 996.4 | 989.4 |
| | | |
| | day 1-RPA | day 1-RPA |
| | 0.49878 | 0.498740554 |
| | 0.50284 | 0.502770781 |
| | 0.50196 | 0.498438287 |
| AVERAGE | 0.50120 | 0.5000 |
| SD | 0.00214 | 0.0024 |
| RSD% | 0.42632 | 0.4838 |

- Precision data of **repeatability**, **intra-day and inter-day reproducibility** of standard solutions, and spiked plasma samples of the drug were all summarized in the Table below:
- The repeatability, intra-day, and inter-day %RSD values for the standard solutions of the drug in the mobile phase were 0.42632, 0.4197, and 0.4438, while the %RSD in the spiked plasma sample was 0.4838, 0.4805, and 0.4766.
- According to the standard guidelines, the proposed analysis method is precise since the values of **%RSD** for all were less than **2.0%.**

| Sample Type / Precision | In Mobile Phase | | In Spiked Plasma Sample | |
|---|---|---|---|---|
| | Mean RPA ± SD | RSD% | Mean RPA ± SD | RSD% |
| Repeatability | 0.5012 ± 0.00214 | **0.42632** | 0.500 ± 0.0024 | **0.4838** |
| Intra day | 0.5011 ± 0.0021 | **0.4197** | 0.50 ± 0.0024 | **0.4805** |
| Inter day | 0.500 ± 0.0022 | **0.4438** | 0.4985 ± 0.0024 | **0.4766** |