Chapter 6

Development and validation of HPLC method for quantification of compound GC466 in rat plasma and brain

6 Introduction

In the previous chapter, a novel GCase chaperone called GC466 [N-[(3-bromo-4-methoxyphenyl) methyl] cycloheptanamine hydrobromide] was identified. GC466 was found to strongly bind to specific residues of rat GCase (Asp146, Trp198, Tyr263, Phe265, Tyr331, His329, Glu358, and Trp399) with a binding energy (BE) of -8.92±0.68 Kcal/mol and binding affinity (Ki) of 0.64±0.12 μM. Its therapeutic potential, including GCase restoration, neuroprotection, and free radical scavenging, has been demonstrated in PD cell line models. To further evaluate the therapeutic potential of GC466 for PD, *in-vivo* studies were conducted using an anti-PD model in rats. The results showed that GC466 exhibited neuroprotective effects by enhancing GCase activity and associated cell protective mechanisms, such as reducing endoplasmic reticulum stress (ERS) and inhibiting α-synuclein aggregation in the PD model. Given its promising therapeutic potential in the central nervous system (CNS), it is imperative to conduct comprehensive investigations in the brain (Tripathi, Ganeshpurkar et al. 2023).

To date, the pharmacokinetic properties of GC466 have not been evaluated in preclinical model. However, pharmacokinetic data are crucial for understanding the drug's elimination from the body, its half-life, clearance, and optimizing dosage regimens. Without this information, it is challenging to predict the duration of drug presence in the body or its elimination process (Thummel, Shen et al. 2006). In our previous study (Chapter 3), we utilized *in silico* ADMET tools to identify the ADMET properties of GC466. The results indicated its potential CNS penetration ability. However, to establish the reliability of these *in silico* findings, it is crucial to validate GC466's CNS penetration ability using an *in vivo* preclinical model. Presently, no method exists for assessing the CNS penetration ability of the GC466 compound. Therefore, the primary objective of this

study is to validate an HPLC method for accurately determining the concentration of GC466 in rat plasma and cerebrospinal fluid, as well as to evaluate its distribution in brain tissue. The brain tissue regions selected for this study were the substantia nigra and striatum. These regions were specifically chosen due to their significance as primary targets for the majority of drugs used in the treatment of PD patients. By accomplishing these objectives, we aim to provide robust evidence to validate the *in silico* predictions made regarding GC466's CNS penetration ability.

6.1 Materials and methods

6.1.1 Experimental Chemicals

GCase-acting compoundGC466 (MolPort-029-998-466) was procured from the Molport database (https://www.molport.com/shop/indexI. High-performance liquid chromatography (HPLC) grade water (Merck), acetonitrile (Merck India), dimethyl sulfoxide (DMSO) (Merck, India), thiopentone sodium (Neon laboratories, India), 0.45µm filters (Rankem laboratories, India), were obtained.). Ambroxol (Internal standard) as a gift sample was obtained from (Meenaxi Pharma, Delhi). The rest of the reagents were all of the analytical grade.

6.1.2 Animals

Study was performed in healthy adult male Wister rats (weight range 200–250 g, 8-12 week old) which were obtained from the IIT-BHU, Varanasi, India. Rats were kept in laboratory conditions, with free access to food and water and a 12-hour light-dark cycle. Before the study began, the Institutional Animal Ethics Committee approved the experimental procedures IIT (BHU) IAEC/2023/034. Specifically, the National Institutes of Health's guide for the care and use of laboratory animals, as outlined in NIH

Publications No. 8023 (revised in 1978), was strictly adhered to in the execution of every experiment.

6.1.3 Preparation of stock solution

Primary stock solutions of GC466 and internal standard (IS) were prepared by dissolving the compounds in acetonitrile to achieve desired concentration of 1.0 mg/mL.

6.1.4 Preparation of working solution

The working solution was prepared by carefully diluting the stock solution with acetonitrile, resulting in solutions that encompassed a concentration range of 1 to 500 µg/mL for both GC466 and IS. These concentrations were precisely set at 2.5, 25, 50, 100, 250, and 500 µg/mL. These expertly prepared working solutions served as the foundation for generating the standards calibration curve, ensuring accurate and reliable measurements for further analyses.

6.1.5 Preparation of calibration curve and quality control samples

The calibration and quality control (QC) samples were prepared using blank plasma and tissue homogenates. To obtain plasma, heparinised blood (25 IU/mL) was centrifuged at 4000rpm for 10 minutes at 4°C. Brain tissue homogenates were prepared by adding ice-cold phosphate buffer saline solution to brain tissue at a ratio of 2:1 (mL: g) and then homogenized (Yang, Faustino et al. 2005). For the calibration samples of GC466, concentrations of 0.05, 0.5, 1, 2, 5, and 10 µg/ml were prepared by spiking a specific volume of corresponding working solutions into 90 µl of blank plasma/CSF/tissue homogenates. To each sample, 10 µL of internal standard solution (with a final concentration of 1 µg/ml) was added and vortexed. Protein precipitation was achieved by adding 390 µl of acetonitrile to the samples, which were then capped, vortexed, and centrifuged at 13000 rpm for 10 minutes at 4 °C. The clear supernatant was carefully

transferred to 0.5-mL amber conical vials placed inside white Teflon holders and subsequently injected into the HPLC system. To ensure quality control, additional QC samples were prepared using blank plasma, CSF, and brain homogenates at very low (0.05 µg/mL), low (0.15 µg/mL), medium (1 µg/mL), and high (8 µg/mL) concentrations. These QC samples were stored at − 70 ± 10 °C until analysis. Prior to injection into the HPLC system, the samples were filtered using 0.20 µm nylon filters.

6.1.6 Sample preparation or Extraction of GC466 compound

A simple protein precipitation method with some modifications was employed for the extraction of GC466 from rat plasma, cerebrospinal fluid (CSF), and brain tissues (Yang, Faustino et al. 2005, Chen, Jin et al. 2022, Ramakrishna, Jain et al. 2022). Initially, 90 µL of plasma, CSF, or brain homogenate was taken, to which 10 µL of the internal standard (IS, final concentration 1 µg/ml) solution was added. The mixture was thoroughly mixed for 15 seconds on a cyclomixer (Spinix Tarsons, Kolkata, India). Subsequently, the sample was subjected to extraction using 400 µL of acetonitrile at 13000 rpm for 10 minutes at 4°C. Following centrifugation, the supernatant was collected and further processed. To ensure purity, the supernatant was filtered through 0.22 µm nylon filters. Finally, 5 µL of the filtered sample was injected into the high-performance liquid chromatography (HPLC) system for analysis.

6.1.7 Method validation

6.1.7.1 Linearity and range

A calibration curve was constructed using a range of five concentrations, spanning from 0.05 to 8.00 µg/ mL in blank plasma, cerebrospinal fluid (CSF), and tissue homogenates from both the striatum and nigra regions. To ensure accuracy and reliability four independent runs were performed. In this process, the analyte / IS (internal standard) peak

area ratios were plotted against the corresponding concentrations. To assess the linearity of the data, a comprehensive linear regression analysis was employed, employing the esteemed least square regression method to calculate the regression parameters (Bhandari and Kaur 2012, Ramakrishna, Jain et al. 2022).

6.1.7.2 Precision and accuracy

We conducted a thorough assessment of intra-day and inter-day assay precision and accuracy to ensure the reliability of our results. In the intra-day analysis, we examined four replicates at four distinct QC levels, specifically 0.05, 0.15, 1, and 8.0 µg/mL, all within the same day. To gauge the inter-day assay precision and accuracy, we analyzed the QC samples at the same four levels across four separate runs, spanning three different days. Our acceptance criteria were set to maintain high-quality data, requiring accuracy within the range of 90% to 110%. However, for precision the relative standard deviation (R.S.D.) to fall within ±5% (Mercolini, Mandrioli et al. 2009, Bhandari and Kaur 2012).

6.1.7.3 Limit of detection (LOD) and limit of quantification (LOQ)

The limits of detection (LOD) and quantification (LOQ) play crucial roles in analytical chemistry, enabling the determination of the lowest concentrations of a substance that can be reliably detected and accurately measured within specific experimental conditions. To calculate these parameters, the following formulas are commonly employed:

$$LOD = \frac{3.3\ X\ SD}{S}$$

$$LOQ = \frac{10\ X\ SD}{S}$$

In these formulas, SD refers to the standard deviation of the response (peak area), while S represents the slope of the calibration curve (Mercolini, Mandrioli et al. 2009).

6.1.7.4 Stability

The stability of GC466 in rat plasma, cerebrospinal fluid (CSF), and brain tissues was assessed using three quality control (QC) concentrate levels, namely Low Quality Control (LQC), Medium Quality Control (MQC), and High Quality Control (HQC). Each level was tested in triplicate samples. Various stability parameters were evaluated, including bench-top stability (at room temperature for 4 hours), autosampler stability (placed in the automatic sampler for 24 hours), and freeze-thaw stability (subjected to three freeze-thaw cycles at -20°C for 24 hours each, followed by thawing at room temperature for 1 hour). To determine stability, the assay values were compared to acceptable limits of ±15% Relative Standard Deviation (R.S.D.). Samples were considered stable if the assay values fell within this acceptable range (Khan, Zuthi et al. 2016, Ramakrishna, Jain et al. 2022).

6.1.7.5 Recovery

The recovery of GC466 and IS was assessed using a protein precipitation procedure. To determine the recovery, the responses of the analyte extracted from replicate QC samples (n=3) were compared with the response of analyte from neat standard samples containing equivalent concentrations. For GC466, the recovery was evaluated at three different concentrations: QC low, QC medium, and QC high, which were 0.05, 1, and 8.0 µg/mL, respectively. To assess the acceptability of the recovery, the mean recovery should fall within the range of 90-110%(Yang, Faustino et al. 2005, Khan, Zuthi et al. 2016).

6.1.8 Pharmacokinetic study in rats

A study was conducted on healthy adult male Wister rats to investigate the effects of GC466. The rats, weighing between 210 to 230 grams and aged 8 to 12 weeks, were obtained from IIT-BHU in Varanasi, India. They were housed in laboratory conditions, providing them with unrestricted access to food and water, and maintaining a 12-hour light-dark cycle. Prior to commencing the study, the experimental procedures were

approved by the Institutional Animal Ethics Committee under protocol number IIT(BHU)IAEC/2023/034. To ensure acclimatization, the rats were given a two-week period before the start of the experiments. The sample size for the study was determined through a preliminary G* power analysis. A total of 6 rats were deprived of food with free access to water for 12 h before the experiments, and 25 mg/ kg of GC466 (freshly prepared in water) were orally administered to rats. Blood samples were collected from the tail vein at 0.5, 1.0, 3.0, 6.0, 12.0, and 24.0 h after oral dosing in microfuge tubes containing heparin (25 IU/mL) (Chen, Jin et al. 2022). The plasma sample were then prepared and subjected to the extraction process of the GC466 compound, as detailed in **section 6.1.6**, titled "Sample preparation or Extraction of GC466 compound.

6.1.9 Brain distribution and penetration ability study

A total of 30 rats (n=5 for each time points) were deprived of food with free access to water for 12 h prior to the experiments. The animals acclimatization and sample size was determined as described in section 6.2. GC466 was freshly prepared in water and administered orally to the rats at a dose of 25 mg/kg. At each time points following dosing (0.5, 1.0, 3.0, 6.0, 12.0, and 24.0 hours), four rats were sacrificed. CSF collection was performed under mild anaesthesia (40 mg/kg, *i.p.*) (Ramakrishna, Jain et al. 2022) prior to euthanizing the animals. After the collection of cerebrospinal fluid (CSF), the animals were euthanized in accordance with ethical guidelines. Subsequently, striatum and nigra regions were carefully collected at the specified time points mentioned above. The tissue samples were rinsed with ice-cold 0.9% NaCl solution to remove blood or content and blotted on flter paper. Samples were stored at−80 °C until subsequent processing. The CSF and tissue homogenates samples were then prepared and subjected to the extraction process of the GC466 compound, as detailed in **section 6.1.6**, titled "Sample Preparation or Extraction of GC466 Compound." The experimental procedure can be visually

understood by referring to F**ig. 6.1**, which provides a clear illustration of the entire protocol.

6.1.10 Parameters of plasma pharmacokinetic, brain distribution, and brain penetration

Maximum plasma concentrations (C_{max}) and time to reach the maximum plasma concentration (T_{max}) values were obtained directly from the plasma concentration-time curves of GC466. PK Solver software (Brand, Orr et al. 2013) was used to measure the pharmacokinetic parameters, including area under the curve (AUC), mean residence time (MRT), and half-life (t1/2). To see the brain penetration ability AUC brain to plasma ratio was also determined.

6.2 Data analysis.

Pharmacokinetic parameters and brain penetration data are expressed as mean ± SD. Graph-Pad Prism version 5.1 (San Diego, CA, USA) was used for data analysis. PK Solver software (Brand, Orr et al. 2013) was used to measure the pharmacokinetic parameters.

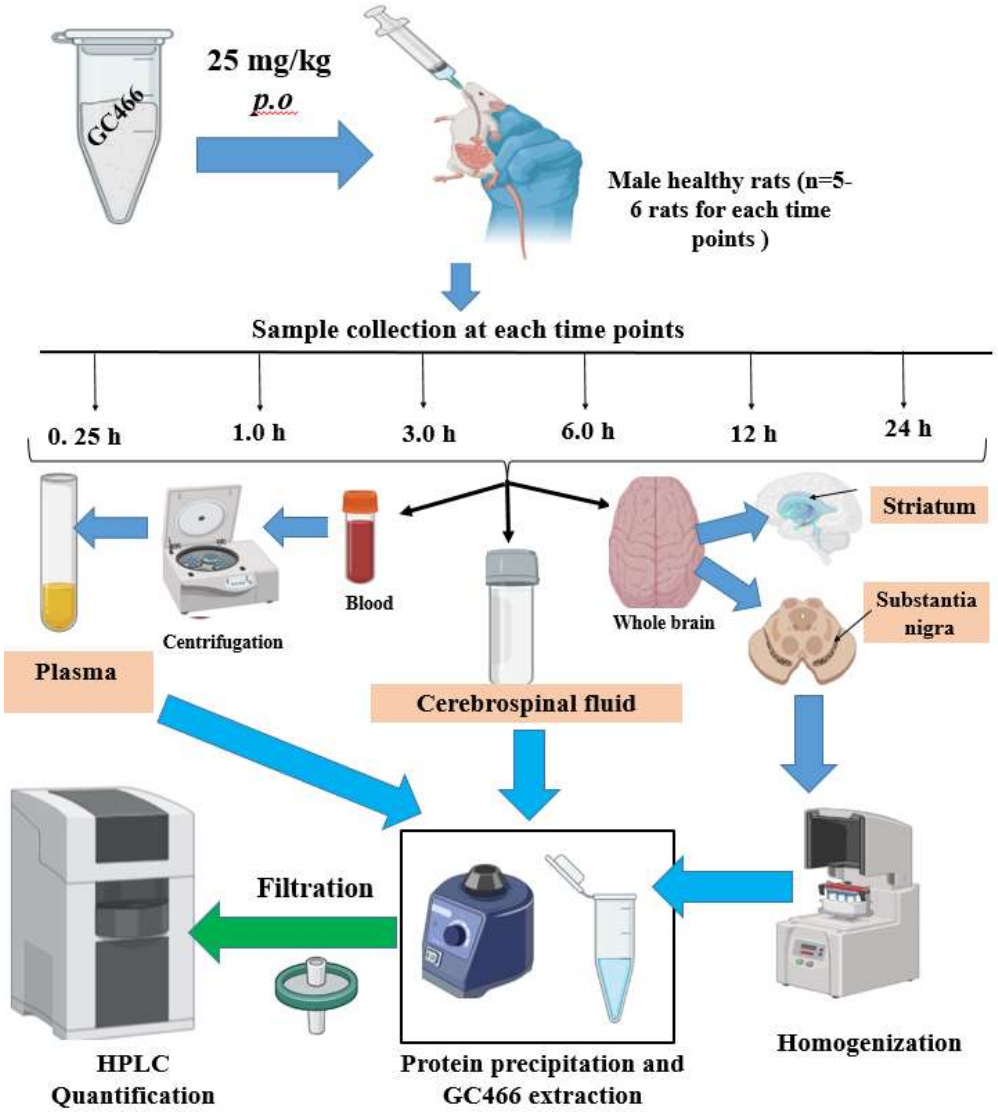

Figure 6.1 Experimental design of the Pharmacokinetic and brain distribution study

6.3 Results and Discussion

6.3.1 HPLC method development and validation

The HPLC method (reversed phase) for quantifying GC466 was meticulously developed and rigorously validated employing equipment from Agilent Technologies, specifically the Infinity 1260 II system. This advanced setup encompasses a degasser, a quaternary pump, and an autosampler, all of which work seamlessly together. Additionally, the system features a highly sensitive photodiode array (PDA) detector and is driven by the

Agilent open LAB CDS software, a powerful tool for data acquisition and processing. Method development began with the optimization of chromatographic conditions such as mobile phase. The analytes of interest, GC466, was quantified using the PDA detector at a wavelength of 278 nm, providing optimal sensitivity for accurate measurements. A high high-quality C18 column measuring 4.6 x 250 mm (5µm particle size) with mobile phase consisting of 0.1M Acetate buffer, pH 5.0 (A) : acetonitrile (B) (30:70, v/v) at a flow rate of 0.7 mL/min provided the best compromise between selectivity, sensitivity and speed of analysis. The retention times of GC466 and internal standard were found to be 4.158 and 5.279 min, respectively. The overall analysis time was only 6.0 min. In order to validate a HPLC method following parameters were evaluated

6.3.1.1 Assessment of selectivity, linearity, and range

The calibration curve exhibited a remarkable linear trend across the range of concentrations tested for plasma, CSF, and tissue homogenate, as evidenced by correlation coefficients (r2) exceeding 0.998. The correlation coefficients (r2) obtained were consistently higher than 0.995, suggesting a strong association between the measured concentrations and the corresponding responses. The slope (m) and intercept (c) and r2 of the linear calibration curves are mentioned in the **Table 6.1**. However, in the selectivity we found that the sample was found to be effective and reliable for estimation of GC466 in both plasma and brain matrices. No interfering peaks were seen at the retention times of GC466 or IS in both the matrices.

Table 6.1 Linearity and range of the developed method

S. No	Sample description	Concentration range (µg/ml)	Equations	r2
1	Plasma	0.05 to 8	Y = 0.4397X + 0.2302	0.998

2	Striatum (ST)	0.05 to 8	Y = 0.5855X + 0.3686	0.997
3	Substantia nigra pars compacta (SNp)	0.05 to 8	Y = 0.5479X + 0.3049	0.995
4	Cerebrospinal fluid (CSF)	0.05 to 8	Y = 0.5096X + 0.2714	0.998

6.3.1.2 Precision and accuracy determination

The tables in this study, **Tables 6.2**, provide valuable insights into the accuracy and precision of data obtained from various samples, including plasma, cerebrospinal fluid (CSF), and brain tissue (ST and SNp). The results indicate that the accuracy and precision fell within an acceptable range of 90.4 - 100.72% and 0.0080- 2.612, respectively. These findings establish that the assay values obtained for both intra- and inter-day measurements remained well within acceptable limits. These results ensuring the reliability and validity of HPLC analyses.

6.3.1.3 Determination of Limit of detection (LOD) and limit of quantification (LOQ)

The LOD represents the minimum concentration in a sample that can be detected, though not necessarily quantified. On the other hand, the LOQ represents the lowest concentration of an analyte that can be determined with acceptable precision and accuracy. Both were used to indicate the sensitivity of the method. The limit of detection (LOD) values for GC466 were determined to fall within the range of 0.011 µg/ml to 0.014 µg/ml in plasma, cerebrospinal fluid (CSF), and brain tissues. Additionally, the limit of

quantification (LOQ) was found to be within the range of 0.026 µg/ml to 0.029 µg/ml in plasma, CSF, and brain tissues.

Table 6.2 Precision and accuracy of the chaperone GC466

Regions		0.05 µg/ml	1.00 µg/ml	8.00 µg/ml
Plasma	**Intra-day variation**			
	Cal. Conc.	0.048	0.986	7.867
	SD	0.0024	0.0443	0.0680
	Precision (%)	0.0080	1.5872	0.4789
	Accuracy (%)	96.00	98.6	98.34
	Inter-day variation			
	Cal. Conc.	0.046	0.922	7.442
	SD	0.0013	0.0435	0.0192
	Precision (%)	1.075	0.8130	1.667
	Accuracy (%)	92.00	92.2	93.02
Striatum	**Intra-day variation**			
	Cal. Conc.	0.049	0.966	7.738
	SD	0.0023	0.0765	0.0451
	Precision (%)	1.0617	0.1465	0.2092
	Accuracy (%)	98.20	96.6	96.72
	Inter-day variation			
	Cal. Conc.	0.048	0.935	8.146
	SD	0.0035	0.0190	0.0483
	Precision (%)	1.0397	1.8274	0.9324
	Accuracy (%)	96.00	93.5	100.72
(SNp)	**Intra-day variation**			
	Cal. Conc.	0.047	0.985	7.956
	SD	0.0031	0.0058	0.0204
	Precision (%)	1.2073	0.6454	1.4437
	Accuracy (%)	94.2	98.5	99.45
	Inter-day variation			
	Cal.Conc.	0.045	0.926	7.738
	SD	1.0617	1.1456	0.2092
	Precision (%)	2.612	0.1931	0.6488
	Accuracy (%)	90.4	92.61	92.25
CSF	**Intra-day variation**			
	Cal.Conc.	0.051	0.996	7.940
	SD	0.0045	0.0254	0.0609
	Precision (%)	1.2679	2.5533	2.4018
	Accuracy (%)	102.0	99.6	99.25
	Inter-day variation			
	Cal. Conc.	0.046	0.953	7.425
	SD	0.0021	0.0383	0.0548
	Precision (%)	0.7954	0.2703	1.3422

Accuracy (%)	92.4	95.3	92.83

Calculated concentration (Cal.conc.), standard deviation (SD). All precision and accuracy values run in n=4.

6.3.1.4 Determination of stability

The stability tests conducted on plasma and tissue samples consistently showed that the target compounds remained stable throughout the entire process of sample storage and chromatographic analysis. This method proves to be highly suitable for the routine analysis of GC466 in plasma, cerebrospinal fluid (CSF), and brain tissues (Yang, Faustino et al. 2005). The experimental findings unequivocally indicated that the chaperone GC466 did not undergo any degradation under any of the tested conditions, as demonstrated in **Table 6.3**. The extraction of GC466 from plasma, CSF, and tissue homogenates was efficiently achieved through a straightforward deproteination process using ACN, enabling a simple, convenient, and rapid separation of the analyte. The quality control (QC) samples of GC466 exhibited stability in both plasma and tissue homogenates, with results falling within the acceptable range of precision ±15%. The performance evaluation of GC466 demonstrated a remarkable level of stability, with accuracy consistently falling within a range of 90% to 99%.

Table 6.3 Stability of GC466 at different conditions

		Plasma			Striatum			SNp			CSF		
Benchtop	**Known conc.**	**Cal. Conc.**	**Pre. (%)**	**Acc. (%)**	**Cal. Conc.**	**Pre (%)**	**Acc. (%)**	**Cal. Conc.**	**Pre. (%)**	**Acc. (%)**	**Cal. Conc.**	**Pre (%)**	**Acc. (%)**
	0.05	0.048	1.45	96.00	0.049	1.06	98.00	0.046	2.73	92.00	0.047	2.76	94.2
	1	0.972	1.21	97.2	0.987	4.53	98.7	0.911	0.83	91.11	0.959	4.63	95.9
	8	7.951	2.54	99.39	7.548	1.39	94.35	7.647	1.24	95.58	7.7487	3.84	96.85
Freeze-thaw stability	0.05	0.044	3.76	94.00	0.048	1.06	96.00	0.051	2.83	99.2	0.049	1.58	98.2
	1	0.922	1.56	92.2	0.976	2.19	97.6	0.098	0.945	98.00	0.9094	1.84	90.94
	8	7.540	0.57	94.25	7.9481	0.73	99.35	7.8472	1.11	98.09	7.851	1.84	98.14
Auto-sampler	0.05	0.052	2.75	99.90	0.047	1.65	94.2	0.049	4.76	98.2	0.046	2.56	92.5
	1	0.914	0.53	91.4	0.975	1.33	97.5	0.994	2.84	99.4	1.073	2.16	97.3
	8	7.354	0.68	91.93	7.432	1.79	93.00	7.576	1.55	94.70	7.983	4.39	99.78

Known concentration (Known conc.), Accurcay (Acc), Precision (Pre). All values represented as mean (n=3).

6.3.1.5 Determination of recovery

Recovery in HPLC refers to the measurement of the efficiency of a particular method to extract, separate, and detect a target analyte. It provides insights into the accuracy and reliability of the HPLC method being employed (Yang, Faustino et al. 2005, Chen, Jin et al. 2022). By determining the recovery, analysts can validate the entire analytical process and ensure the consistency and reproducibility of the results obtained. The recovery of GC466 from the quality control samples is depicted in Table **6.4.** The recoveries of GC466 from plasma, CSF and tissue samples ranged from 91.53 to 99.64 % which fell in acceptable ranges.

Table 6.4 Recovery of GC466 in plasma, CSF and brain tissue (Striatum and Nigra).

Con.	Plasma		Striatum		Substantia nigra pars compact		Cerebrospinal fluid	
(µg/ml)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
0.05	93.16	2.07	95.73	1.23	92.95	1.27	91.53	1.55
1	91.95	3.48	99.23	0.18	99.16	0.51	92.43	3.55
8	92.67	1.57	99.64	0.23	97.98	0.26	99.53	1.36

R = Percentage Recovery; RSD = Relative standard deviation (RSD). All values are expressed as mean (n=3).

6.3.2 Plasma pharmacokinetic profile in rats

The mean plasma concentration–time profiles following administration of GC466 at a dose of 25 mg/kg orally are depicted in **Figure 6.2. Table 6.5** presents the pharmacokinetic parameters of GC466 in the bloodstream. The maximum observed concentration (C_{max}) was 0.745 ± 0.012 µg/mL, reached at a time (T_{max}) of 0.685 ± 0.044 h. These findings indicate that GC466 was rapidly absorbed, with the maximum plasma

concentration achieved approximately 41 minutes after oral administration. This rapid absorption can be attributed to the lipophilic nature and better permeability of GC466. The lipophilicity of GC466 is substantiated by a previous study in which a LogP value of 4.8 was observed (Tripathi, Ganeshpurkar et al. 2023). The distribution half-life (t1/2,α) was found to be 0.45 ± 0.07 h, while the mean area under the plasma concentration-time curve ($AUC_{0–24}$) was determined to be 4453.83 μg/mL *h. The data presented strongly suggest that GC466, when administered orally, exhibits a rapid distribution within the plasma, reaching detectable levels within a remarkable timeframe of just 27 minutes.

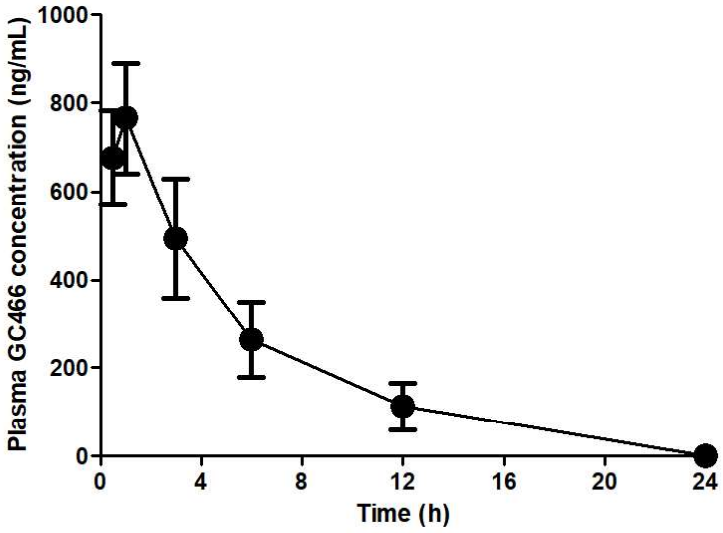

Figure 6.2 Mean ± SD (n=6) plasma concentration–time curves of GCase chaperone GC466 after 25 mg/kg single oral administration.

However, following absorption and distribution, GC466 exhibited an exponential decline with an elimination half-life (t1/2, β) of 3.16 ± 0.42 h, suggesting a slower elimination phase and an optimal duration of action. These results align with a previous study where AMB (GCase chaperone) also displayed an exponential decline with a half-life (t1/2) of 3.124 ± 2.395 h in rats treated with a dose of 6.3 mg/kg (Zhou, Zhang et al. 2021). The

mean residence time (MRT) of GC466 was calculated as 4.43 h, indicating an average time of 4.43 h that GC466 remains in the body. In comparison, ambroxol, an existing GCase chaperone administered at a dose of 6.3 mg/kg in rats, reached a plasma concentration of only 86.45 ± 18 ng/mL with a mean residence time of 2.91 hours, as reported previously (Zhou, Zhang et al. 2021). Based on the findings of this study, it can be inferred that the exposure time of GC466 in the plasma is significantly longer compared to existing AMB. Consequently, frequent dosing may not be necessary when using GC466. **Figure 6.3 (a)** showing the typical chromatogram of plasma sample after oral administration of GC466.

Table 6.5 Plasma pharmacokinetic parameters of glucocerebrosidase chaperone GC466 after 25 mg/kg single oral administration in rats

1	Parameters	Unit	GC466
2	C_{max}	µg/ml	0.745 ± 0.012
3	T_{max}	h	0.685 ± 0.044
4	$t_{1/2}\,\alpha$	h	0.45 ± 0.07 h
5	$t_{1/2}\,\beta$	h	3.16 ± 0.42
6	$AUC_{(0-24)}$	ng /ml*h	4453.83
7	$AUC_{(0-\infty)}$	ng /ml*h	4479.68
8	MRT	h	4.43

.All values are Mean ± SD (n=6).

6.3.3 Brain penetration ability and tissue distribution study of GC466 in rats

The rat brain samples obtained after administering GC466 (25 mg/kg) orally were subjected to analysis using a developed and validated method. The concentration-time

profiles of GC466 in different brain regions are presented in **Figure 6.4**. The maximum concentrations of GCase chaperone GC466 in brain tissues were measured as 0.173 ± 0.011 µg/mL, 0.227 ± 0.047 µg/g, and 0.204 ± 0.021 µg/g for cerebrospinal fluid (CSF), striatum (ST), and substantia nigra pars compacta (SNp), respectively. Interestingly, the striatum exhibited higher concentrations compared to other brain regions, which could be attributed to the higher expression of GCase in this area, as supported by the earlier study (Gegg, Burke et al. 2012). **Fig. 6.3 (b)** showing the typical chromatogram of striatal brain sample after oral administration of GC466. The time taken by GC466 to reach its maximum concentration (T_{max}) in various brain regions, such as CSF, ST, and SNp, were found to be 2.19 ± 0.16 h, 2.43 ± 0.12 h, and 2.68 ± 0.17 h, respectively. These findings suggest that GC466 required approximately 130 to 160 minutes to be absorbed into the tissue after crossing the blood-brain barrier and reaching their peak concentrations. The elimination half-life (t1/2) of GC466 was determined to be 4.07 ± 0.86 h, 4.76 ± 0.84 h, and 4.82 ± 1.02 h for CSF, ST, and SNp, respectively, indicating, the elimination of GC466 from the brain is slower in comparison to plasma. From this we can also inferred that the presence of GC466 in the brain longer period of time as compared to plasma.

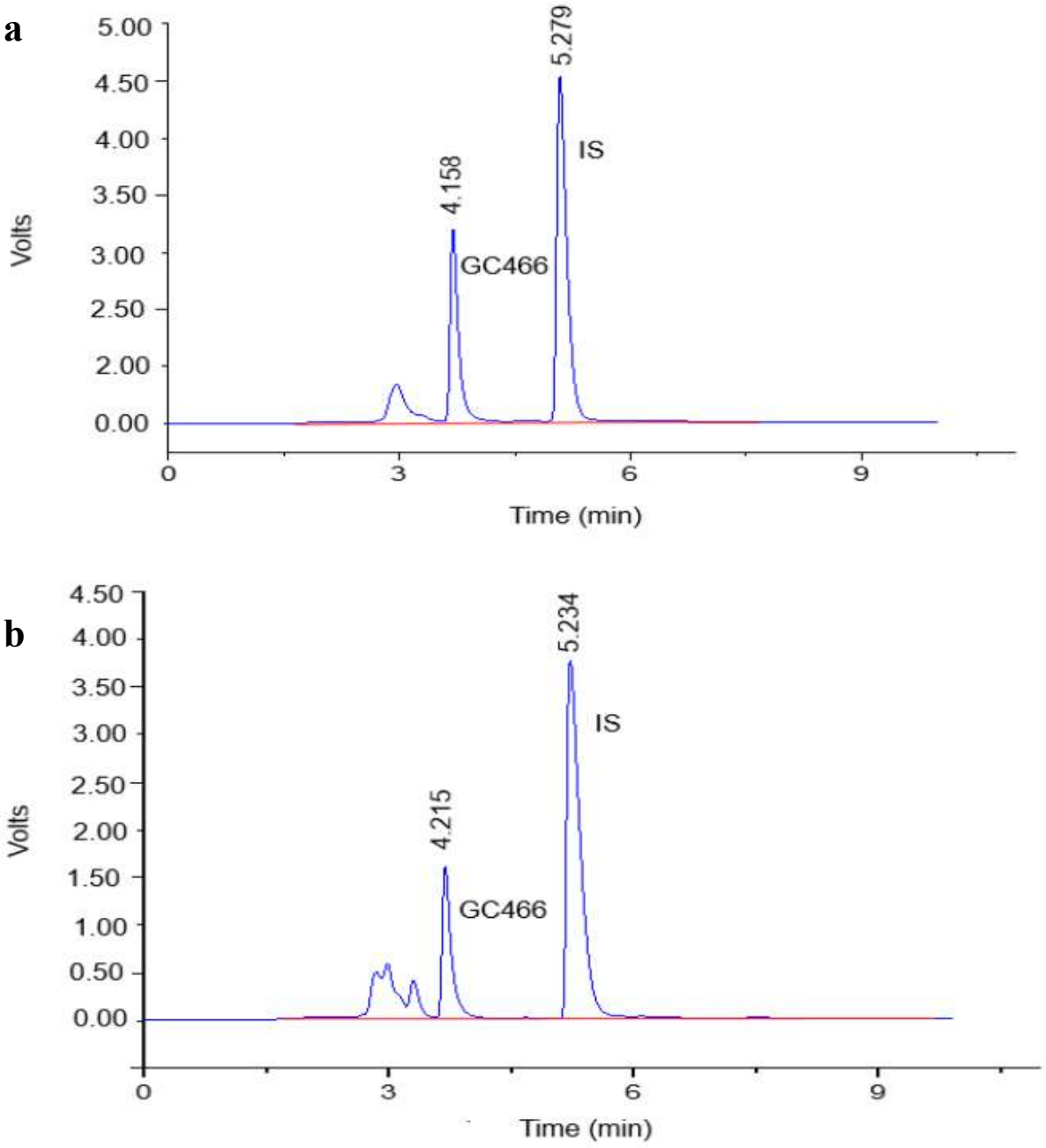

Figure 6.3 Typical chromatograms of a plasma sample (a) and a striatal (target brain tissue where it is found more) brain sample (b) from rat treated with oral dose of GC466. "IS" denotes internal standard (Ambroxol).

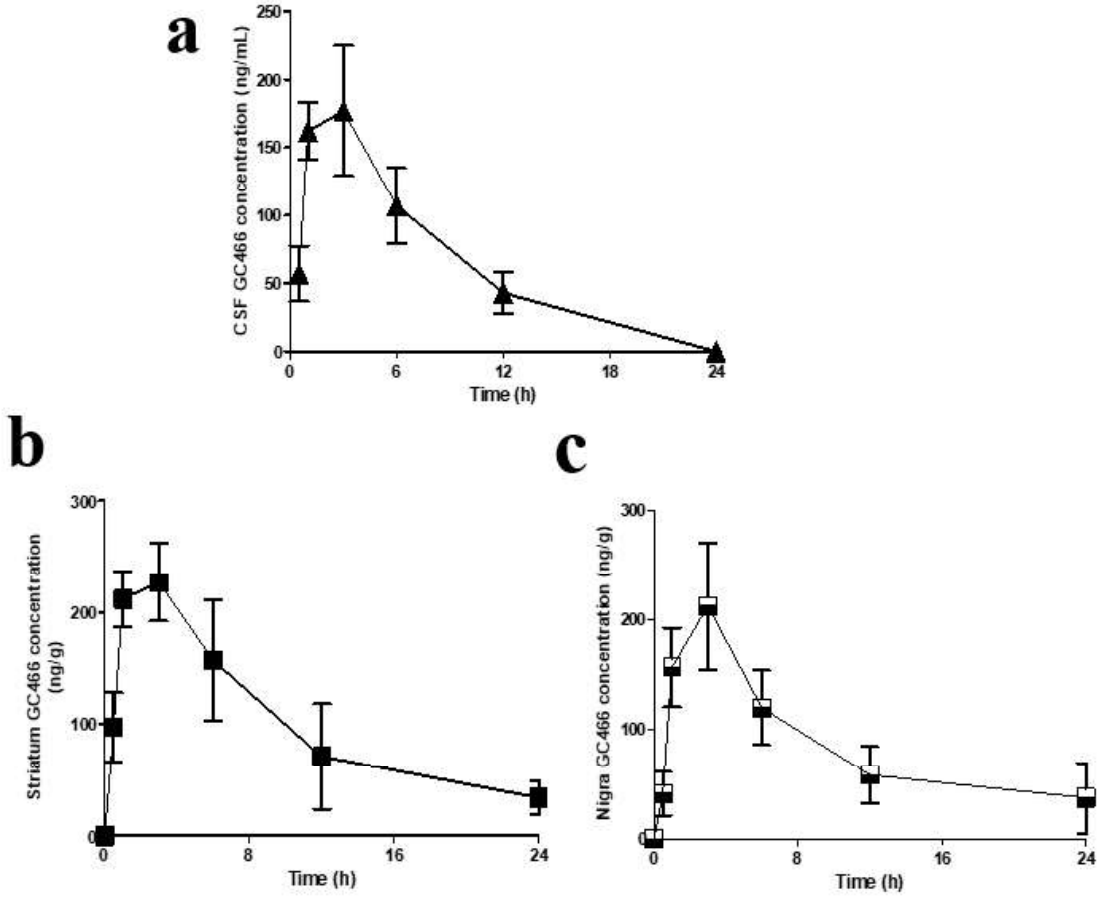

Figure 6.4 GC466 concentration in different brain regions such as CSF (a), striatum (b), and substantia nigra pars compacta (SNp, c) following oral administration at 25 mg/kg. All values are indicated as Mean ± SD (n=5).

Mean Residence Time (MRT) is an important pharmacokinetic parameter that helps assess the overall duration of drug exposure in the body. It provides valuable insights into dosing frequency and optimal dosage regimens for a drug. In this study, the MRT values for CSF, ST, and SNp were found to be 6.94 ± 1.23 h, 8.16 ± 1.36 h, and 8.22 ± 1.13 h, respectively. These data indicate that the brain exposure of GC466 is approximately twice as high as in the plasma, suggesting a longer duration of GC466 presence in the brain target area where glucocerebrosides (target for PD) are present, compared to plasma. Furthermore, in the brain penetration study, we calculated the AUC Brain/AUC Plasma

ratio. The AUC represents the overall exposure to a drug over a specific period of time, and the AUC Brain/AUC Plasma ratio compares the drug's distribution in the brain to its concentration in the plasma. The ratios for CSF, ST, and SNp were found to be 0.552, 0.604, and 0.501, respectively. These values indicate that approximately 50-60% of the drug's exposure in the plasma is also observed in the brain tissue, suggesting good brain penetration.

6.4 Conclusion

A validated HPLC method was meticulously developed to effectively monitor the presence of the GCase chaperone, GC466, in both the bloodstream and brain of rats. Through this research endeavor, we sought to assess the plasma pharmacokinetics and the extent to which GC466 traverses the blood-brain barrier after oral administration at a dose of 25 mg/kg. Remarkably, our research stands as the first to demonstrate the successful and efficient traversal of the blood-brain barrier by GCase chaperone, swiftly reaching target regions within the brain, namely, the CSF, ST, and SNp. Following oral administration, the drug exhibited rapid absorption, achieving peak plasma concentrations within a mere 41 minutes. Brain penetration study indicate that the approximately 50-60% of the drug's exposure in the plasma is also observed in the brain striatum and nigra tissues, suggesting good brain penetration. Notably, the duration of action of GC466 observed in the striatum and nigra tissues—known as the primary targets for Parkinson's disease—were approximately one and half times as high as those found in the plasma. This significant finding suggests an extended duration of GC466 presence in these regions, in comparison to its presence in the plasma. When contemplating the collective impact of all these factors, it becomes increasingly evident that a substantial quantity of GC466 effectively permeates the intricate confines of the brain. Such a

remarkable observation potentially elucidates the previously reported neuroprotective properties associated with this remarkable compound.

6.5 Summary

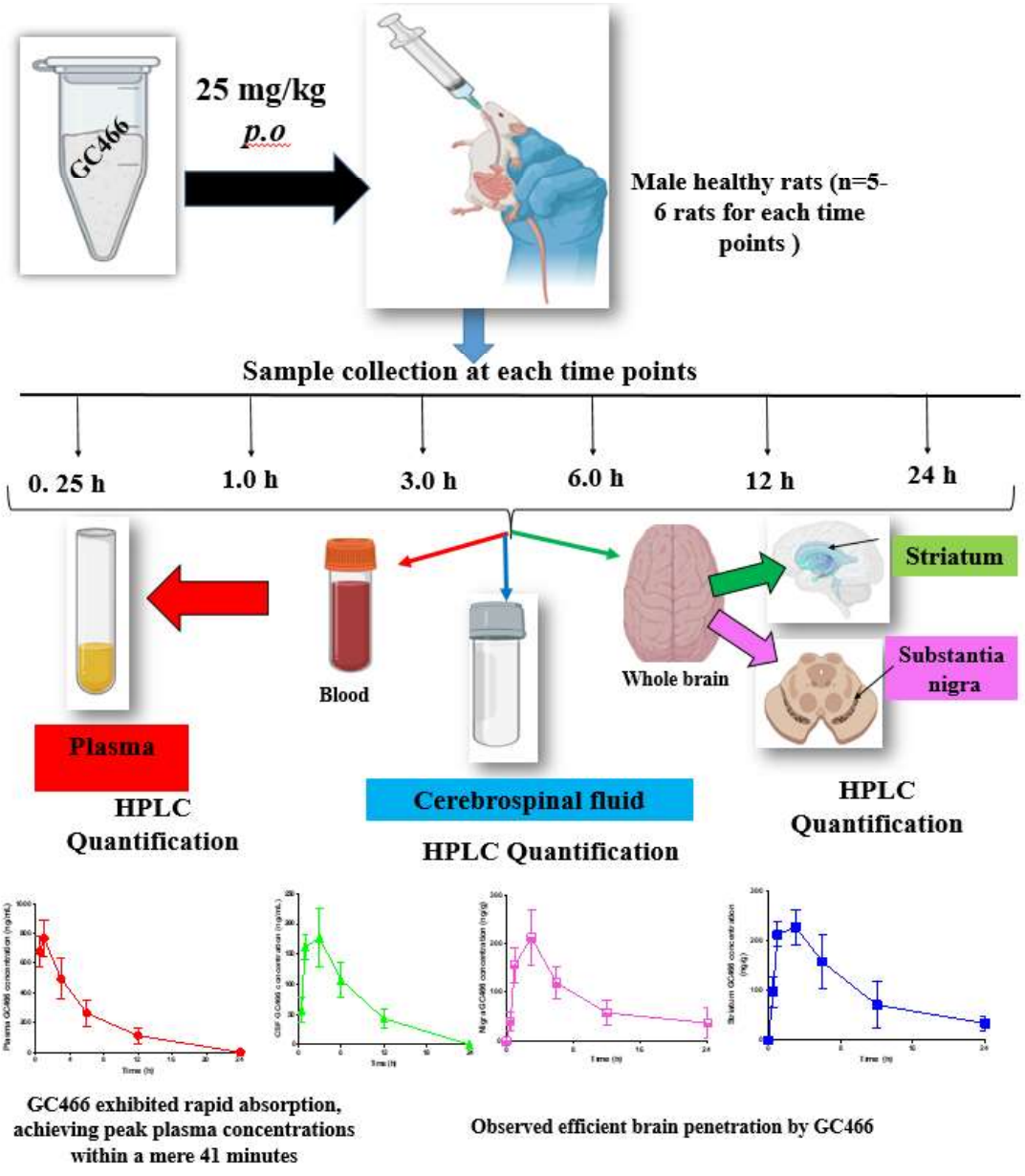

Fig. 6.6 summarizes the experimental outcomes of the pharmacokinetics and brain distribution study.

- GC466, in both the plasma and brain regions of rats are measured at an oral dose of 25 mg/kg.
- GC466 exhibited rapid absorption, achieving peak plasma concentrations within a 41 minutes.
- GC466 required approximately 129 to 144 minutes to cross the brain.
- 50-60% of the drug's exposure in the plasma is also observed in the brain striatum and nigra tissues by GC466, suggesting good brain penetration.