RESEARCH ARTICLE

A Comprehensive Report on the HPLC-ESI-MS/MS Method Development and Validation for Quantifying Cabotegravir in K_3EDTA-Human Plasma, Employing Cabotegravir-D_5 as an Internal Standard

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ABSTRACT

Aiming to comprehend the plasma concentration of cabotegravir, a meticulously crafted bioanalytical method, aligned with the guidelines set by the US Food and Drug Administration (FDA), was devised and validated. This involved the utilization of cabotegravir D5 as an isotopic internal standard. The mobile phase consisted of methanol and 5 mM ammonium acetate in water, in an 80:20 ratio, v/v—Zorbax SB-C18 (50 × 2.1 mm, 5 µm) served as the chromatographic column. Cabotegravir and cabotegravir D5 were identified using proton adducts at m/z 409.20/370.20 and 409.20/144.8, respectively, employing the positive mode multiple reaction monitoring. Cabotegravir exhibited linearity within concentration ranges of 2 to 1000 ng/mL (r^2 = 0.999). The recovery of cabotegravir from plasma by Liquid-liquid extraction yielded an average %CV of 7.71 at four quality control levels. Intra-day precision displayed accuracy of 100, 106, 97, 97, and 103%, respectively, whereas accuracy for inter-day precision was 100, 103, 98, 100, and 100% across LLQC, LQC, MQC-1, MQC-2, and HQC levels. The stability of cabotegravir remained consistent under diverse conditions, including five freeze-thaw cycles, benchtop, autosampler, and short-term, and long-term storage. This evaluation verifies that the method aligns with predefined acceptance limits and positions it as an indispensable tool in bioanalysis, significantly expanding its clinical utility.

Keywords: Cabotegravir, Cabotegravir D5, Human plasma, LC-MS/MS, Isotopic internal standard.

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INTRODUCTION

In the year 2021, the global count of new human immunodeficiency virus (HIV) cases remained static at 1.5 million, mirroring the statistics of the preceding year. This apparent stabilization underscores a plateau in prevention initiatives, signaling a critical need for enhanced strategies. The daily incidence of new HIV infections worldwide stood at 4,000, predominantly affecting specific demographics, notably sex workers, who represented 70% of these cases. The implementation of highly effective antiretroviral therapy has had a profound impact on patients living with HIV in terms of both survival duration and quality of life. While notable advancements have been made, it remains crucial to adhere to daily antiretroviral therapy steadfastly. This adherence is essential for sustaining virological suppression, preventing drug resistance, and mitigating the potential for HIV transmission.". The potential for long-lasting exposures at therapeutic concentrations of long-acting antiretrovirals could facilitate the simplification of treatment regimens, eliminating the need for daily administration.[1] Due to its distinctive physiochemical and pharmacokinetic characteristics, cabotegravir (CBT), with the chemical designation N-((2,4-difluorophenyl) methyl)-6-hydroxy-3-methyl-5,7-dioxo-2,3,5,7,11,11a hexahydro (1,3) oxazolo(3,2- a)pyrido(1,2-d) pyrazine-8-carboxamide, has been developed and delivered in two distinct formulations: A sustained-release nanosuspension formulated for intramuscular use at intervals spanning from monthly to quarterly. Furthermore, findings derived from challenge models involving simian/human immunodeficiency virus in nonhuman primates and vaginal CBT long-acting suggest that it may serve as a viable option for the prevention of HIV[2-4] potentially offering a practical substitute for

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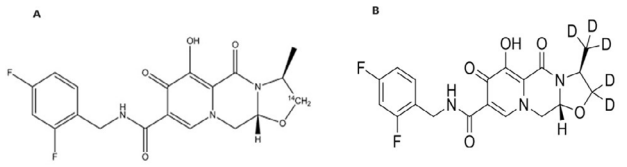

Figure 1: Molecular structure of A) Cabotegravir B) Cabotegravir-D5

preexposure prophylaxis against HIV.[5] The unique two-drug combination consisting of CBT and rilpivirine, administered orally which has also been formulated for long-acting injectable administration, has proven to be an effective treatment for HIV. It enables antiretroviral-naivepatients to maintain viral suppression.[6] CBT targets the pivotal HIV integrase enzyme, impeding the transfer and integration of the virus into the host genome.[7] Despite its high effectiveness and minimal safety concerns, caution and extensive evaluation, particularly among populations like pregnant individuals and those historically excluded from research, remain imperative. There are still lingering worries about these treatments, particularly about how well patients stick to the treatment, the potential harm the drug may cause, and how easy it is to take the prescribed doses.[8] Hence, there is a strong need to close the aforementioned gaps in knowledge and advancement in clinical studies of CBT for the prevention and treatment of HIV-1 infection. Effective analytical techniques for the detectionof CBT are in high demand. When combined with rilpivirine, sophisticated analytical techniques, including liquid chromatography-mass spectrometry (LC-MS), high-performance liquid chromatography (HPLC), and ultra-performance liquid chromatography (UPLC) could provide accurate CBT measurement.[9-21] Apart from a singular LC-MS method utilized to estimate CBT,[22] there's currently no established liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique documented for the quantification of CBT utilizing isotopic internal standards. Hence an ingenious LC-MS/MS method has been devised to identify CBT, with CBT-D_5 serving as an isotopic internal standard with similar chemical traits to ensure efficient extraction and recovery rates. Figure 1 illustrates the structures of both CBT and CBT-D5. The findings of these methods may be applied to expanding CBT's therapeutic applicability.

MATERIALS AND METHODS

Materials and Compounds

A sample of CBT with a purity level of 99.87% and CBT-D5 (used as an internal standard) with a purity of 99.94% were acquired from Aurobindo Pharma Ltd. in Hyderabad, India. Various chemicals and reagents, including analytical-grade ammonium acetate, LCMS-grade methanol, and Milli-Q water purification system-purified water obtained exclusively from the Merck chemical division in Mumbai, India, met the quality standards necessary for HPLC analysis. HPLC-grade water was consistently maintained at the highest purity level throughout the investigation. A sample of human K3-EDTA Plasma was acquired from the St. Theresa blood bank in Hyderabad, India, ensuring adherence to ethical protocols.

LC-MS/MS Instrument Specifications and Parameters

In the pursuit of quantification of CBT, the LC-MS/MS system, anchored by the Shimadzu LC VP Series was employed. This instrumental platform featured a high-speed autosampler, column oven, and degasser. The analytical precision of this LC-MS/MS methodology was elevated by the incorporation of an MDS Sciex API 4000 mass spectrometer. Data processing was executed using the Analyst version 1.4.2 and Watson LIMS version 7.3 software. The detection of target compounds was accomplished through electrospray ionization (ESI) with the utilization of the turbo ion spray feature, operating in the positive ionization mode and affording unit resolution. The employment of the multiple reactions monitoring (MRM) mode is renowned for its superior specificity and sensitivity. In the case of CBT, the precursor ion MH^+ (m/z 404.20), and a selected product ion at m/z 374.20 acted as guiding beacon. CBT-D5 with its precursor ion MH^+ (m/z 409.20) and corresponding product ion at (m/z 379.20) served as reference points. To optimize the mass spectrometric parameters, the source temperature was set to an elevated 500°C, while the heater gas was maintained at 21 psi (nitrogen)to ensure efficient ionization. The nebulizer gas, operating at 31 psi (nitrogen), facilitated a controlled sample introduction, and the curtain gas, set at 20 psi (nitrogen), acted as a protective shield for the analytical environment. The collision-activated dissociation (CAD) gas, at 6 psi (nitrogen), played a pivotal role in enhancing fragmentation, while the ion spray (IS) voltage remained constant at 5000 V, ensuring a consistent ionization source. The source flow rate was held at 600 ml/min without splitting, preserving the integrity of the analyte, and the entrance potential was judiciously set to 10 V for smooth ion transition. Both the analyte and the ISTD shared a decluttering potential of 125 V, facilitating efficient ion separation. Furthermore, the collisionenergy was consistently maintained at 35V for both the substance of interest and the internal reference, ensuring effective fragmentation. The exit potential of the collision cell was universally set at 15 V for both analyte and internal standards.

Chromatographic Conditions

The analytical column utilised in the chromatographic analysis was a Zorbax SB-C_{18} (50 × 2.1 mm, 5 µm) column manufactured by Agilent Technologies. The mobile phase consisted of a blend of methanol and water with 5 mM ammonium acetate., with a volumetric ratio of 80:20. A diluent was used, comprising a 50:50 v/v mixture of methanol and HPLC grade water. With the aid of a splitter, the mobile phase was introduced into the system at a rate of 0.8 mL/min. The column temperature was maintained consistently under ambient conditions for the duration of the analysis. CBT-D5 was identified as a suitable internal standard after careful evaluation of its extractability and chromatographic performance. The volume of injection utilised for each sample was 15 µL. CBT exhibited a retention time of around 2.65 ± 0.3 minutes, while CBT-D_5 showed a

retention time of roughly 2.62 ± 0.3 minutes. Consequently, the combined duration of each analysis was summed up to approximately 3 minutes.

Preparation of Calibration Standards and Quality Control Samples

A stock solution of CBT (1-mg/mL) was meticulously prepared by dissolving precisely measured quantities in the specified diluent. Calibration Standards and Quality Control samples were generated by fortifying blank plasma with precise volumes of working solutions derived from intermediate stock solutions for both analytes. The resulting CBT concentrations encompassed 2, 4, 10, 25, 50, 100, 125, 250, 500, and 1000 ng/mL. QC samples were prepared at six levels, specifically 2 ng/mL (LLoQ), 6 ng/mL (LQC), 50 ng/mL (MQC-1), 400 ng/mL (MQC-2), 800 ng/mL (HQC), and 1000 ng/ml (ULoQ). A distinct stock solution for the internal standard, CBT D5 (1-mg/mL), was freshly prepared on the analysis day by dissolving accurately measured quantities in the diluent. The working solution of internal standard derived from their stock solutions in diluent (7095.24 ng/mL), was utilized for spiking and stored at 2 to 8°C until use. Standard stock and working solutions were reserved for spiking and stored at 2 to 8°C, whereas CSs and QC samples in plasma were stored at -70°C.

Sample Retrieval Protocol

By utilising liquid-liquid extraction, CBT and CBT-D_5 were extracted from plasma samples. All frozen CSs and QC samples were defrosted and permitted to stabilize at ambient temperature before extraction. In 15 mL pre-labeled glass containers, after precisely transferring 250 µL of plasma via pipette, 25 µL of ISTD (7095.24 ng/mL) was introduced and the mixture was vortexed for 20 seconds. Before being agitated for 10 minutes, 100 µL of 0.1% formic acid buffer was added to this. With the addition of 5 mL of tert-butyl methyl ether, the mixture was vigorously agitated at 200 rpm for 20 minutes. The organic phase, located at the upper layer (4.0 mL), was carefully transferred to a designated glass test tube and subjected to drying for 25 minutes at 40°C under a mild nitrogen stream after centrifugation at 4000 rpm for 10 minutes at 4°C. The resulting residue was then reconstituted in 500 µL of the mobile phase using 3 minutes of vortex mixing at 3000 rpm. Following this, the final reconstituted sample was transferred to a labeled glass autosampler vial, and 15 µL of the sample was injected into the chromatographic unit.

Validation of the Developed Method

Linearity

Nine calibration standards were created by introducing working standard solutions and the ISTD into control rat plasma, covering from 2 to 1000 ng/mL. Through analysis of data collected from preliminary investigations, a calibration curve was established. This curve was developed by plotting the back-calculated concentration against the nominal concentration across thirteen analytical runs. Each calibration curve underwent individual assessment using a least square weighted ($1/x^2$) linear regression method, chosen for its ability to best align with the concentration-detector response relationship. It was required that the retro-calculated concentrations of all standards for the calibration curve fall within ± 15 % of their nominal concentration, with a widened margin of ± 20% specifically at the LLoQ. Additionally, a minimum of 75 % of the total calibration curve standards is needed to satisfy the established norms of acceptance, encompassing a minimum of one standard each for the LLoQ and ULoQ.

Precision and accuracy

At least 67% of the QCs should be within ± 15% (± 20 % for LLOQ) of their nominal concentration. At least 50% of the QCs at each level should be within ± 15% (± 20 % for LLoQ) of their nominal concentration. Intra-batch precision and accuracy were assessed by rigorous evaluation of each concentration at the LLoQ (2 ng/mL), LQC (6 ng/mL), MQC-, (50 ng/mL), MQC-2, (400 ng/mL), and HQC (800 ng/mL) using six replicates on the same day. For inter-batch accuracy and precision, 24 replicates of sampleswere analyzed at each of the five concentrations. The obtained response and the expected true response at the five quality control levels were compared to calculate %CV and accuracy based on the area response ratio values for both inter and intra-batches.

Selectivity assessment

The assessment of selectivity was conducted by establishing criteria wherein the peak area of the target compound, at its designated retention time within the blank samples, was mandated not to exceed 20% of the mean peak area of the LLoQ of CBT**.** Correspondingly, for CBT-D_5 the peak area at its retention time in blank samples was not to surpass 5% of the mean peak area of the LLoQ of CBT. Method selectivity was evaluated using 10 distinct sources of human plasma-seven being normal and the remaining three being lipidemic, hemolyzed, and heparinized. These samples were subjected to analysis both in their blank states and as spiked LLoQ samples. The observed area response in blank samples was compared against the mean area response of LLoQ samples. Furthermore, specificity was determined by examining screened plasma samples that were spiked with coadministered drugs, including paracetamol, dicyclomine, nicotine, pantoprazole, ibuprofen, caffeine, diphenhydramine, and pseudoephedrine.

%Recovery

In the evaluation of the extraction recovery of CBT and ISTD from plasma, a meticulous process involved examining six replicated injections of quality control samples comprising LQC, MQC-1, MQC-2, and HQC, were set at concentrations of 6, 50, 400, and 800 ng/mL, respectively. This determination was established by comparing the peak areas extracted from plasma samples against those obtained from a standard solution spiked with the residue of blank plasma. To meet the criteria, the %CV for replicates at each QC level was required to fall within 15%. Moreover, the %CV for the %recovery across all concentration levels was to be within 15%.

Stability

Under the specific guidelines outlined by the FDA, the stability of an analyte is considered satisfactory if the observed alteration in its concentration remains below a 15% threshold. Freeze-thaw stability evaluation for CBT in plasma was conducted, affirming stability after at least five freeze-thaw cycles at temperatures ranging between -20 ± 50°C and -78 ± 8°C. These samples were then compared against freshly injected internal control samples post the fifth cycle, aiming to ascertain the analyte's resilience against repeated freezing and thawing. In the context of benchtop stability assessment, the stability of CBT in plasma was established for a duration of up to 6 hours at room temperature. Subsequently, these plasma samples were juxtaposed against promptly analyzed plasma extracts. Short-term stability investigations involved preserving six replicates of ULoQ and LLoQ level CBT samples in diluent for 25 hours at room temperature, followed by subsequent analysis. Similarly, in long-term stability assessments, six replicates of ULoQ and LLoQ levels for CBT samples in diluent were stored for a duration of 7 days at 5 ± 3°C. The evaluation of autosampler stability was conducted by maintaining spiked quality control samples at 5 ± 3°C in Ammonium formate for up to 124 hours. This investigation aimed to determine the stability of the analyte when subjected to extended periods within an autosampler. Dry extract stability was examined for 121 hours at temperatures ranging between -20 ± 5°C. Moreover, the durability of the extract at ambient temperature was examined for 4 hours using a mixture of acetonitrile: 20 mm Ammonium formate at an 80:20% v/v ratio. The assessment of the stability of the analyte involved comparing these samples with freshly extracted ones, ensuring that the %RSD remained below 15%.

Matrix Effect

The assessment of the matrix effect's potential influence on the quantification of CBT involves the calculation of a parameter known as the matrix factor (MF). MFs are derived from the discrete measurement of peak area responses for CBT and CBT-D5, which are determined separately. The ratio of these two factors provides the ISTD-normalized MF. When stable-isotope-labeled ISTD is employed, the ISTD normalized MFs should closely approximate unity due to the analogous chemical characteristics and elution durations. It is crucial to emphasize the need for evaluating the matrix effect across various plasma sample types, encompassing lipemic, hemolyzed, and normal K3EDTA plasma specimens. Specifically, four sets of blank biological K3EDTA plasma matrices are meticulously prepared. Each of these setsis subjected to two separate spiking events with a standard solution at four distinct concentration levels: LQC, MQC-1, MQC-2, and HQC levels. Furthermore, two additional plasma samples, characterized by lipemic and hemolyzed attributes, are prepared alongside normal K3EDTA plasma. Each of these sets is subjected to two spike events with the above standard solution followed by a comparison with the clean standards at equivalent concentration levels through alternate injections.

RESULTS

Method Validation

Selectivity assessment

At the retention period of CBT and CBT-D5, it is apparent from Figure 2 that there were no detected interferences in the blank plasma samples injected from different lots. Representative chromatograms of standard zero, LLoQ, and ULoQ samples for CBT are illustrated in Figures 3, 4, and 5, respectively in conjunction with the ISTD.

Accuracy and precision

In the evaluation of intraday accuracy, the accuracy percentages for the specified concentrations across the quality control levels of LLQC, LQC, MQC-1, MQC-2, and HQC were determined to be 100, 106, 97, 97, and 103%. Similarly, in the inter-day precision study, the accuracy percentages for these concentrations were consistently observed to be 100, 103, 98, 100, and 100%. The precision and accuracy were within the pre-specified acceptable limits as shown in Table 1.

Linearity

The validation yielded a linear calibration curve across the concentration spectrum of 2 to 1000 ng/mL, exhibiting a correlation coefficient (r^2) of 0.9999. Each concentration level was individually assessed through least square weighted ($1/x^2$) linear regression, selected for its optimal alignment with the concentration-detector response relationship, as denoted by the mean linear equation y = (0.0021x - 0.0053). Validation of the calibration curves was substantiated by back-calculating the concentrations of CBT and CBT-D5 in human plasma. All derived concentrations fell well within the acceptable thresholds. Additionally, the determined detection limits LoD and LoQ were found to be 12.8 and 38.7 ng/mL, respectively. The linearity results are presented in Table 2.

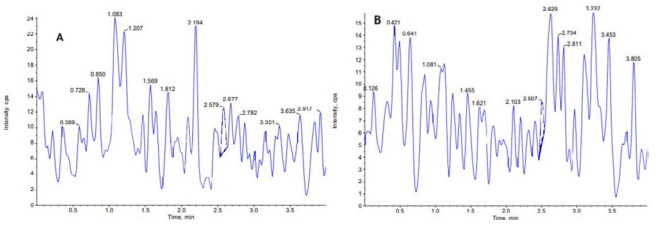

Figure 2: Chromatographic profile of double blank A) Blank – CBT B). Blank – CBT-D5

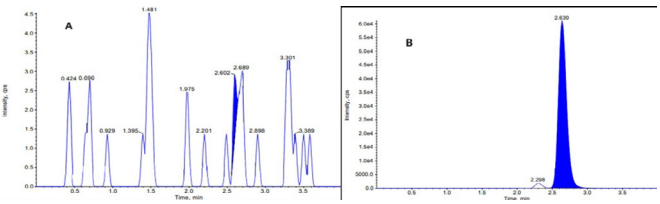

Figure 3: Chromatogram of standard zero A) Blank + ISD-CBT B) Blank + ISD-CBT-D5

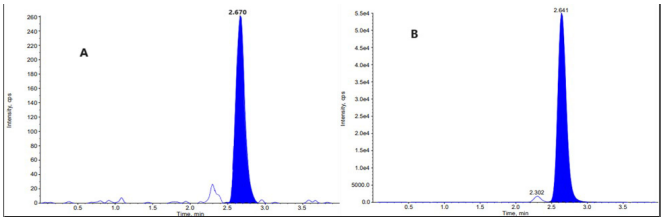

Figure 4: Chromatographic profile of LLoQ A) CBT- Standard (2 ng/mL) B) CBT-D5

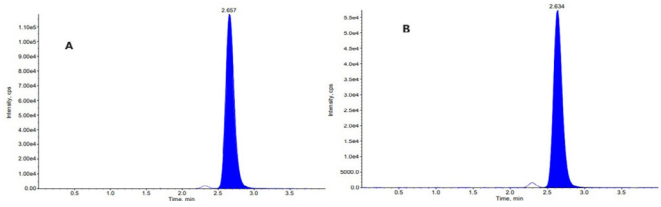

Figure 5: Chromatographic profile of the ULoQ A) CBT- standard (1000 ng/mL) B) CBT-D5

Table 1: Precision and accuracy data for intra-day and inter-day measurements of CBT

Mean concentration ± SD		*%CV*	*Accuracy%*
Intraday precision n = 6 at each QC level			
LLQC	2.00 ± 0.0703	3.52	100
LQC	6.35 ± 0.201	3.17	106
MQC-1	48.5 ± 0.850	1.75	97
MQC-2	386 ± 4.20	1.09	97
HQC	820 ± 13.2	1.61	103
Interday Precision n = 24 at each QC level			
LLQC	1.99 ± 0.150	7.54	100
LQC	6.15 ± 0.318	5.17	103
MQC-1	49.2 ± 1.07	2.17	98
MQC-2	399 ± 9.88	2.48	100
HQC	800 ± 24.2	3.03	100

Table 2: Data of calibration curve for CBT

Nominal concentration ng/mL	*Mean measured concentration ± SD*	*%CVX*	*Accuracy%*
2	2.02 ± 0.15	7.4	101
4	4.01 ± 0.28	6.9	100
10	9.73 ± 0.36	3.7	97
25	24.1 ± 0.69	2.8	96
50	48.6 ± 1.47	3.0	97
125	126 ± 2.80	2.2	101
250	254 ± 5.20	2.0	102
500	511 ± 11.0	2.1	102
1000	1040 ± 27.3	2.6	104

Mean n = The average of 13 determinations

Matrix Effect

Using stable-isotope-labeled ISTD resulted in an ISTD-normalized matrix factor of 0.99, demonstrating proximity to unity. No discernible signal suppression or enhancement was noted at the elution times of CBT and CBT-D5. When tested across eight different plasma lots, it yielded a %CV of 0.56, comfortably meeting the predefined acceptance criterion as indicated in Table 3.

%Recovery

The mean overall recovery rate for CBT reached 86%, coupled with a notably low coefficient of variation of 7.71%, as expressly outlined in Table 4. These findings undeniably validate the effectiveness of the bioanalytical technique in attaining a substantial extraction output.

Stability

The %mean stability range for LQC and HQC samples should ideally fall within 85 to 115% compared to reference samples. Following an extensive benchtop stability examination, the precision of CBT sample accuracy was rigorously assessed, yielding accuracy values of 112.8 and 111.7% for LQC and HQC levels, respectively. Moreover, freeze-thaw stability assessments conducted at LQC and HQC levels indicated accuracy values of 98.5 and 101% at (-20 ± 5°C) and 101 and 101 at (-78 ± 8°C) after undergoing five freeze-thaw cycles. The sustained stability of these samples for a duration of up to 7 days is indicated by the results of short-term stability evaluations with %CV values of 4.35 and 1.45, respectively, and long-term stability evaluations with %CV values of 8.96 and 1.19, respectively at LLoQ and ULoQ, all of which remain within 15% of the established acceptance threshold. Additionally, injection system stability assessments of fortified quality control samples preserved at 5 ± 3°C in the mobile phase demonstrated stability for up to 124 hours without significant loss of CBT, with values of 114 and 110%, affirming the stability of processed samples under auto-sampler conditions compared to freshly prepared counterparts. In conclusion, the comprehensive stability evaluations affirm that the CBT samples consistently conform to the defined variability limits throughout the analytical process.

DISCUSSIONS

Optimizing LC-MS conditions becomes pivotal in the pursuit of streamlined analysis, involving a sequence of deliberate experiments to fine-tune chromatographic parameters and mass spectrometry configurations. This meticulous optimization process encompassed comprehensive adjustments in mass spectrometry settings, specifically executed in the positive turbo ion spray (TIS) mode. Precise calibration was achieved by directly infusing solutions of CBT and CBT-D5 into the TIS source, wherein careful adjustments were made to factors like nebulizers and desolvation gases. These refinements aimed at achieving an optimal spray shape to enhance ionization efficiency and facilitate droplet drying. For the precursor ions of PC, the MH^+ centered at m/z 404.20, with a selected

Table 3: The results of the matrix effect of CBT

Matrix Lot No.	*HQC*			*MQC-1*			*MQC-2*			*LQC*		
	CBT MF	*CBT-D5 MF*	*ISTD NMF*	*CBT MF*	*CBT-D5 MF*	*ISTD NMF*	*CBT MF*	*CBT-D5 MF*	*ISTD NMF*	*CBT MF*	*CBT-D5 MF*	*ISTD NMF*
1	0.94	0.93	1.01	0.83	0.83	1.00	0.92	0.92	1.00	0.87	0.89	0.98
2	0.88	0.87	1.01	0.90	0.90	1.00	0.89	0.90	0.99	0.90	0.92	0.99
3	0.89	0.90	0.99	0.88	0.90	0.98	0.88	0.90	0.98	0.88	0.91	0.96
4	0.92	0.93	1.00	0.93	0.93	0.99	0.95	0.95	1.00	0.95	0.95	0.99
Hemolyzed	0.93	0.94	0.99	0.93	0.93	1.00	0.93	0.94	1.00	0.94	0.94	1.00
Lipidemic	0.94	0.95	0.99	0.94	0.96	0.99	0.97	0.97	1.00	0.94	0.96	0.98
Mean			0.996			0.994			0.995			0.984
SD			0.0096			0.008			0.0056			0.013
%CV			0.97			0.86			0.56			1.33
Mean			0.9922									
SD			0.00557									
%C V			0.56									

MF is the matrix factor and NMF is the normalized matrix factor

Table 4: Study of CBT extraction recovery in human plasma (n = 6)

QC level	*Average extracted peak area ± SD*	*Average un-extracted peak area ± SD*	*%Mean recovery*	*%Mean recovery with correction factor*
HQC	752647 ± 22444	1197655 ± 58717	62.8	78.55
MQC1	410379 ± 22058	575472.5 ± 23857	71.3	89.14
MQC2	52215 ± 1211	69936 ± 2131	74.7	93.33
LQC	5810 ± 689	8807 ± 542	66.0	82.46
		Mean	68.7	85.87
		Overall CV%		7.71

*Mean n = The average of 6 determinations

fragment ion at m/z 374.20, was meticulously calibrated. Similarly, for ISTD, rigorous observation of the precursor ion MH^+ at m/z 409.2 and the daughter ion at m/z 379.2 was conducted. Customizing parameters unique to both the source and compound is pivotal for ensuring consistent and sufficient responses to CBT. A dwell time of 200 minutes was determined as optimal, providing an adequate data point density for CBT quantization while avoiding interference between CBT and ISTD with identical product ions. Deuteration, specifically, has garnered significant interest due to its capacity to influence the way drugs behave in terms of their pharmacokinetics and metabolic attributes. As a result, CBT D5 was deliberately chosen to serve as an internal standard. The pivotal turning point in optimizing chromatographic parameters occurred with the adoption of an eluent mixture comprising methanol and 5 mM ammonium acetate in waterat a volumetric ratio of 80:20. This configuration, coupled with the utilization of a Zorbax SB-C18 (50 x 2.1 mm, 5µm) column, selected for its compatibility with mass spectrometric detection, resulted in enhanced analyte response. In repeated experiments using human plasma, LC-MS/ MS determined the retention times of CBT and CBT-D5 at 2.65 ± 0.3 a n d 2.62 ± 0.3 min, respectively, ensuring a reproducible and reliable analysis free from substances. Importantly, this method exhibited no carryover effect. The calibration standard curve for CBT in human plasma showcased excellent linearity within the range of 2 to 1000 ng/mL ($r^2 = 0.9999$), represented by the equation y= 0.0021x - 0.0053. Stability assessments revealed CBTS stability for 6 hours at normal temperature, through five freeze and thaw cycles, and for 124 hours in autosampler stability at 5 ± 3°C. Accuracy evaluations for CBT in human plasma yielded intraday values of 100, 106, 97, and 103% at LLQC, LQC, MQC, and HQC, respectively. The corresponding inter-day accuracy values were 100, 103, 98, and 100% for four concentration levels (LLQC, LQC, MQC, and LLoQ). In conclusion, an LC–MS/MS method demonstrating reliability in detecting CBT within human plasma has been proposed, aligning with the guidelines set forth by the US FDA. This methodology's credibility encompasses the extraction and analysis of CBT and its internal standard, CBT-D5, in K3 EDTA human plasma samples, covering a concentration range from 2 to 1000 ng/mL. The results and discussions presented indicate that each sample's analysis can be efficiently completed inunder 3 minutes. This developed method stands proficient in estimating low concentrations of CBT in plasma within a single analytical run. These findings hold potential significance in furtheringcomprehension of CBT's pharmacokinetic attributes, thereby enhancing its clinical applications.

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REFERENCES

1. Paterson DL, Swindells S, Mohr J. Adherence to protease inhibitor therapy and outcomes in patients with HIV infection. Annals of Internal Medicine. 2000; 133: 21–30.
2. Radzio J, Spreen W, Yueh YL. The long-acting integrase inhibitor GSK744 protects macaques from repeated intravaginal SHIV challenges. Science translational medicine. 2015; 7(270):270ra5. doi: 10.1126/scitranslmed.3010297.
3. Andrews CD, Yueh YL, Spreen WR. A long-acting integrase inhibitor protects female macaques from repeated high-dose intravaginal SHIV challenge. Science translational medicine. 2015; 7(270):270ra4. doi: 10.1126/scitranslmed.3010298.
4. Andrews C, Spreen W, Mohri H. Long-acting integrase inhibitor protects macaques from intrarectal simian/human immunodeficiency virus. Science. 2014; 343: 1151–1154. doi: 10.1126/science.1248707.
5. Yifan Yu, Kristi B, Mark M, Raphael L, Marybeth M, Susan F. A Population Pharmacokinetic Model Based on HPTN 077 of Long-acting Injectable Cabotegravir for HIV PrEP. British Journal of Clinical Pharmacology. 2022; 88(10):4623-4632. doi: 10.1111/bcp.15477.
6. Scott L, Anthony M, Debbie H, Susan S, Franco F, Jerome D. Pharmacokinetics and antiviral activity of cabotegravir and rilpivirine in cerebrospinal fluid following long-acting injectable administration in HIV-infected adults. Journal of antimicrobial chemotherapy. 2020; 75(I3),648– 655, https://doi.org/10.1093/jac/dkz504
7. Chenzhong L, Christophe M, Terrence RB, Yves P, Marc C Nicklaus A. HIV-1 integrase inhibitors for treating HIV-1/AIDS. Future Medicinal Chemistry. 2010; 2(7): 1107–1122. doi: 10.4155/fmc.10.199. PMID: 21426159; PMCID: PMC3413320.
8. Dario C, Cristina G. Pharmacokinetics and Pharmacodynamics of Cabotegravir, a Long-Acting HIV Integrase Strand Transfer Inhibitor. European Journal of Drug Metabolism and Pharmacokinetics. 2019; 44(3):319-327. doi: 10.1007/s13318-018-0526-2.
9. Anuradha V, Subrahmanyam T, Raju M, Murthy B, Emmanuel K. Method development and validation for Cabotegravir and Rilpivirine by using HPLC and its degradants are characterized by LCMS and FTIR. Future Journal of Pharmaceutical Sciences. 2021; 7(226). DOI: https://doi.org/10.1186/s43094-021-00355-8
10. Aaron RJ, Hutton A, Peng A, Fabiana VZ, Ismaiel A, Tekko AB. HPLC-MS method for simultaneous quantification of the antiretroviral agents rilpivirine and cabotegravir in rat plasma and tissues. Journal of Pharmaceutical and Biomedical Analysis. 2022; 213(114698). doi: 10.1016/j.jpba.2022.114698.
11. Yogi P, Samixa P. RP-HPLC stability method development & validation for anti-HIV drugs cabotegravir & rilpivirine in I.M. injection and in human plasma. International Journal of Health Sciences. 2022; 2550-6978. DOI: 10.53730/ijhs. v6nS1.7056.
12. Padmabhushana CV, Sireesha D. Stability Indicating Reverse Phase-High Performance Liquid Chromatography Method for Simultaneous Estimation of Cabotegravir and Rilpivirine. Indian Journal of Pharmaceutical Education and Research. 2023; 57(3):766-771. doi:10.5530/ijper.57.3s.87.
13. Parthiban C. RP-HPLC Method Development and Validation for the Simultaneous Estimation of Cabotegravir and Rilpivirine in Pharmaceutical Dosage Form. International journal of pharmaceutical education and research.2022; 26(1): 65-79.
14. Suneetha A, Vijaya Lakshmi M, Jyothi k, Priyanka B. Development and Validation of Stability Indicating RP-HPLC Method for the Simultaneous Determination of Cabotegravir and Rilpivirine in Bulk and Injection Dosage Form. Journal of Pharmaceutical Research International. 2022; 21(4) 144-150. DOI: 10.18579/jopcr/v21i4.22.15.
15. Mohammed A, Hemanth KS, Mohammad ZB. Method development and validation for the simultaneous estimation of cabotegravir and rilpivirine in bulk and pharmaceutical dosage form and stability studies by uplc. International Journal of Food Sciences and Nutrition. 2022; 11(1): 2320 –7876.
16. Bhaskar OA, Sunbee P, Vinod AB. Method Development and Validation of Cabotegravir by Using HPLC and Characterization of its Degradants by LC-MS. European Chemical Bulletin. 2023; 12(4) 10597-10611. doi: 10.48047/ecb/2023.12.si4.959
17. Ethel DW, Teresa LP, Ryann G, Marybeth M, Beatriz G, Raphael J. Development and validation of a liquid chromatographic-tandem mass spectrometric assay for the quantification of cabotegravir and rilpivirine from dried blood spots. Journal of pharmaceutical and biomedical analysis. 2023; 10(228). doi: 10.1016/j.jpba.2023.115307.
18. Perrine C, Susana AS, Matthias C, Catia M, Eva C, Chantal C. Development and validation of a multiplex UHPLC-MS/MS assay with stable isotopic internal standards for the monitoring of the plasma concentrations of the antiretroviral drugs bictegravir, cabotegravir, doravirine, and rilpivirine in people living with HIV. Journal of mass spectrometry. 2020; 55(6): doi: 10.1002/jms.4506
19. Inken KR, Marco TA, Lalitkumar K, Aaron RJ, Hutton K, Fabiana VZ. HPLC-MS method for simultaneous quantification of the antiretroviral agents rilpivirine and cabotegravir in rat plasma and tissues. Journal of pharmaceutical and biomedical analysis. 2022; 213. 114698. doi: 10.1016/j.jpba.2022.114698.
20. Sailaja O, Krishnaveni G, Swathi K. A Modern Method for Analyzing Related Substances of Cabotegravir and Rilpivirine Using RP-HPLC, along with the Characterization of their Degradation Products Via LCMS. Asian Journal of Pharmacy. 2023; 17 (2): 300
21. Bevers LAH, Ewijk EW, Brake HML, Burger DM. Development, validation, and clinical implementation of a UPLC-MS/MS bioanalytical method for simultaneous quantification of cabotegravir and rilpivirine E-isomer in human plasma. Journal of pharmaceutical and biomedical analysis. 2024; 238: 115832.
22. Sankar P, Naresh P. Stability Indicating LC-MS/MS Method Development and Validation for theQuantification of Cabotegravir in Biological Samples. International journal of pharmaceutical quality assurance. 2023; 14(03):751-755. DOI:10.25258/ijpqa.14.3.49.