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Solid phase extraction: Potential carbocisteine bioanalysis using carbocisteine 13C3 stable isotope: LC-MS/MS technique with pharmacokinetics on healthy human subjects

Rajesh Dhiman*, Vijaya Durga[2], Jayasankar K. and Sakthi Raj

Micro Therapeutic Research Labs Pvt. Ltd. Chennai, Tamilnadu, India

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ABSTRACT

High Performance Liquid Chromatographic tandem mass spectrometric method for the estimation of Carbocisteine in human plasma has been developed and validated using Carbocisteine 13 C 3 as internal standard. Sample preparation process was accomplished by solid phase extraction technique. The processed sample was chromatographed and analyzed on Luna 5u HILIC 200 A (150 x 4.6 mm) column using mobile phase [Acetone-M: Buffer-1(40:60% v/v)] and diluent as [Acetone-M: Water (90:10% v/v)] .Carbocisteine was chromatographed and analyzed by MS Detector. The analytical method described is valid the determination of Carbocisteine (over a range of 52.27 µg/ml to 5301.83 µg/ml) using as Carbocisteine 13 C 3 internal standard in human plasma. Signal from the detector were captured in a computer and processed using MassLynx SCN 4.1 v software. This method was found suitable to analyze human plasma samples for the application in pharmacokinetic and BA/BE studies.

Key words; Carbocisteine, Carbocisteine 13C3, LC-MS/MS, and Pharmacokinetics etc.

INTRODUCTION

Carbocisteine is chemically described as (2R)-2-amino-3-[(carboxymethyl) sulfanyl] propanoic acid (Figure 1) is a mucolytic expectorant. It diminishes the viscosity and increases the volume of pathologically thickened sputum, thereby facilitating expectoration [1]. In clinical practice carbocisteine has gained acceptance and is used in the management of respiratory diseases characterized by accumulation of excessive secretions [1]. Carbocisteine is administered orally in liquid or solid dosage forms including syrup, tablet and capsule.[2] Carbocisteine is rapidly well absorbed after oral administration and the subsequent kinetics fit a one-compartment open model.[1,3] Peak serum concentrations are reached between 1 and 2.0 hr and peak values were 10.8 to 13.9 mg/L after a 1500 mg dose[3,4]. Cmax of 8.2 µ/mL at 3.0 hr was observed after administration of 750 mg of carbocisteine in capsule [5]. Cmax of about 13.0 µg/mL was observed after administration of 1000 mg of carbocisteine in granule or suspension [6]. The plasma half-life was estimated to be 1.33 hr and the apparent volume of distribution was approximately 60 L. There is no information on intravenous studies to allow bioavailability determination [1]. There is no reported work on first-pass metabolism or protein binding [1]. Carbocisteine appears to penetrate well into lung tissue [7] and respiratory mucus, [8] suggesting local action. There are no data to suggest a relevant relationship between concentration and effect [1]. Significant variation between the patterns of metabolism in humans and animals has been noted [9, 10]. There are no reports of pharmacologically important activity in these metabolites. The majority of the drug is eliminated unchanged by urinary excretion [9, 10]. Only limited methods have been reported in the HPLC and GC. The objective of the work was to develop and validate LC-MS/MS method for quantification in

Human Plasma. The method shows more sensitive limit of detection and Limit of Quantification is very less to the previous reported methods.

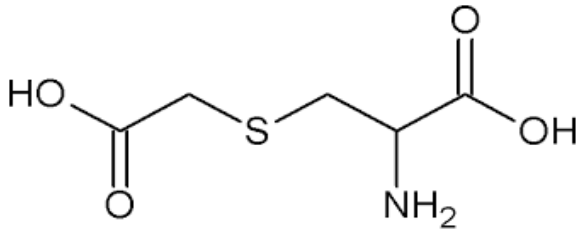

Figure 1: Structure of Carbocisteine

MATERIALS AND METHODS

Chemicals and reagents

Carbocisteine with purity 98.87% w/w (Figure 1) Carbocisteine 13 C 3 and as internal standard with purity 99.24% w/w working standards were used. HPLC grade Acetone-M was manufactured by Qualigens Fine Chemicals. Formic acid AR grade manufactured by S.D. Fine chemicals, Human Plasma (K_2EDTA), Plastic container, Propionic anhydride (GR Grade), Perchloric acid (GR Grade), Liquid ammonia (GR Grade) and Water HPLC grade from Milli-Q RO system was used throughout the analysis.

Preparation of Reagents

Buffer-1- (0.5% formic acid in water), Mobile Phase-Acetone-M: Buffer-1 (40:60, v/v), Diluent- Acetone-M: Water (90:10, v/v), Reconstitution solution (1% Propionic Anhydride in acetone-M), Rinsing Solution-Acetone-M: Water (70:30, v/v), Strong Wash Solution-Acetone-M: Water (90:10, v/v), Weak Wash Solution-Acetone-M: Water (50:50, v/v), Washing Solution- (0.2 % Formic Acid in Water) and Elution Solution-2% Ammonia in Acetone-M (v/v) were used.

Instrumentation and Chromatographic Conditions

The chromatography was performed on WATERS XEVO TQ-S with cooling auto sampler and column oven enabling temperature control of the analytical column. Luna 5u HILIC 200 A (150 x 4.6 mm) column was employed. The column temperature was maintained at 45 °C and chromatographic separations were achieved with isocratic elution using a mobile phase composed of [Acetone-M: Buffer-1(40:60% v/v)] .The flow rate was set at 1.0 ml/min, run time was 4.00 minutes and retention time for Analyte and IS were 2.0±0.30 min and 2.0±0.30 min respectively. The auto sampler was conditioned at 15°C and the injection volume was 20µl using Auto sampler mode for sample injection.

Compound Setting

Tuning Parameters and MS/MS conditions are given in Table 1 and Table 2.

Table 1: Tuning Parameters

Parameters	Values
Capillary (kV)	1.50
Desolvation Gas (L/Hr)	650
Cone Gas Flow (L/Hr)	150
Desolvation Temp. (^{0}C)	350

Table 2: MS/MS Conditions in LC-MS/MS

Molecule	Parent (m/z)	Daughter (m/z)	D well (sec)	Cone (Volts)	Collision energy (eV)
Carbocisteine	234.20	142.20	0.2	30	10
Carbocisteine 13C3	237.40	143.20	0.2	30	10

Sample Preparation

Vortex the thawed samples to ensure complete mixing of contents. Add 50 µl of internal standard solution (25µg/ml) to all the samples except blank. Pipette 500 µl of sample into respective vials containing internal standard solution and vortex well. Add 50 µl of Perchloric acid to all the samples and vortex. Centrifuge the samples at 14000

rpm for 10 minutes in a refrigerated centrifuge between 2°C to 8°C. Collect the upper layer and keep the samples aside for further processing. Equilibrate MCX 30mg/cc cartridges of required numbers with 1 ml of Actone-M followed 1 ml with washing solution. Load the above collected samples into respective cartridges and wash the cartridges twice with washing solution. Transfer the cartridges to respective labeled RIA vials and elute the sample using 1 ml of Elution solution. Collect the eluted sample and dry all the samples under nitrogen evaporator at 40°C and 15 (psi) pressure. Reconstitute the dried residue with 200 µl of Reconstitution solution. Vortex and keep the samples at room temperature for 20 minutes. Transfer the samples into respective labeled auto-injector vials and load the samples into LC-MS/MS.

Validation Parameters

The Method was validated in accordance with FDA Guidelines [11]. Blank screening (Figure 5), Selectivity, Linearity, Accuracy, Precision, Recovery, Stability (Freeze thaw, bench top, long term, Auto sampler, stock solution at RT and RF) and Matrix effect were performed. Each batch of spiked plasma samples includes one complete calibration curve (consisting of one blank plasma, one blank plasma with internal standard and eight different non-zero concentration samples, six replicates quality control samples includes six replicate quality control samples LLOQ, LQC, INTQC, MQC and HQC (Figures 6-11) except Bench top, freeze thaw stability, long term stability and Auto sampler stability in which four replicate quality control samples were used.

Linearity

The linearity of Carbocisteine was determined by weighted least square regression analysis of standard plot associated with eight point standard curve (Figure 3). The calibration was shown to be linear from 52.24µg/ml to 7075.76µg/ml for Carbocisteine. Best –fit calibration lines of chromatographic response versus concentration were determined by weighted least square regression analysis with weighting factor of 1/concentration. The coefficient of correlation (r^2) was consistently greater than or equal to 0.99 during the course of validation for Carbocisteine, which are within limits.

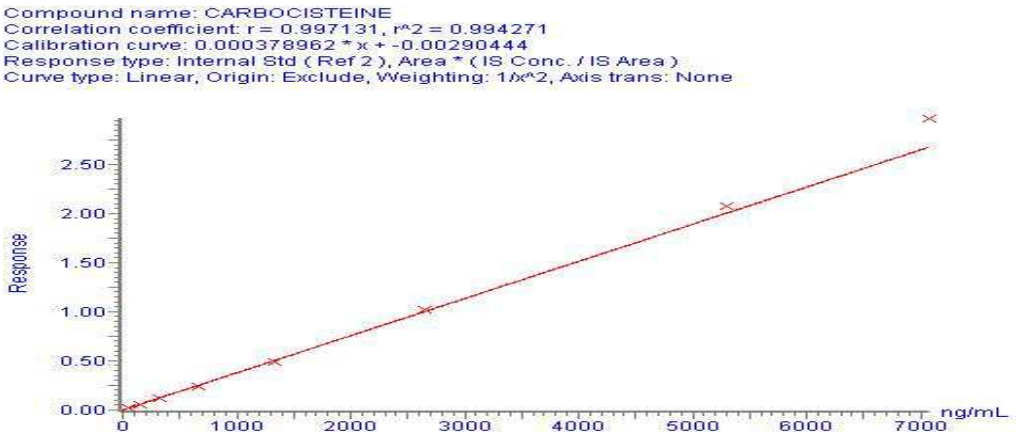

Figure 3: Representative Regression Analysis of a Calibration Curve for Carbocisteine

Specificity and Selectivity

Specificity and Selectivity were evaluated by analyzing a total of nine lots (six lots of blank K_2EDTA human normal plasma, one lot of haemolyzed plasma, one lot of lipemic plasma and one lot of heparin plasma) obtained from independent sources. No significant interferences were observed at the retention times of Analyte and Internal Standard in nine out nine lots evaluated, demonstrating acceptance criteria were met.

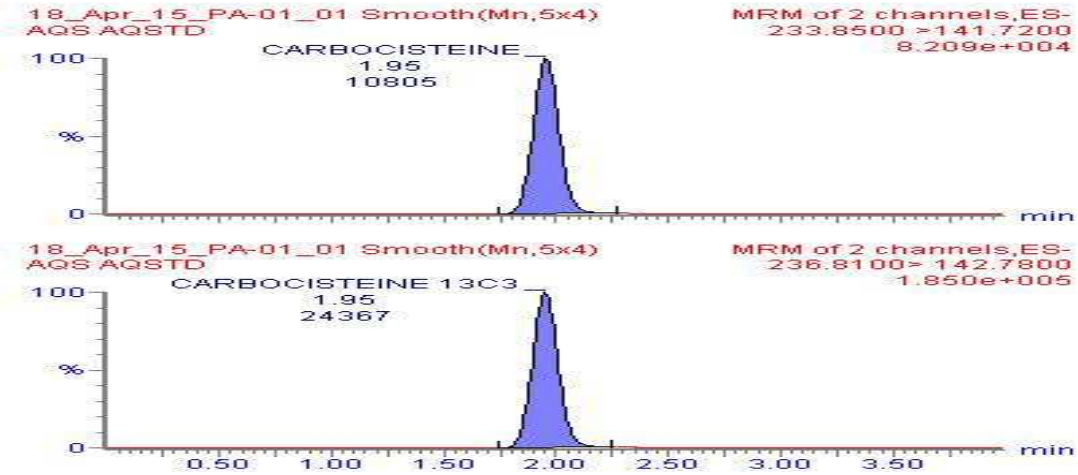

Figure 4: Representative Chromatogram of an Aq. Standard Solution for Carbocisteine

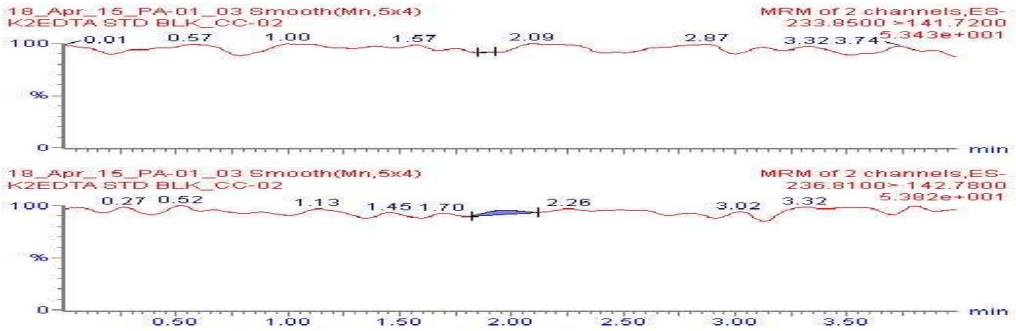

Figure 5: Representative Chromatogram of a Standard Blank for Carbocisteine

Signal-to-Noise (S/N) Ratio

The signal-to-noise ratio was determined at Carbocisteine LLOQ concentrations in nine independent lots of K_2EDTA human normal plasma including one lot of haemolyzed plasma, one lot of lipemic plasma and one lot of heparin plasma. Signal-to-Noise ratios ranged from 632.090 to 3485.809 and 20.761 to 420.197, respectively across the matrix lots evaluated, demonstrating acceptable S/N intensity.

Carry Over Test

Carry over is calculated as the percentage peak area observed in a processed blank plasma injected immediately after a processed ULOQ calibration standard used from Precision and Accuracy batch samples. No significant carry over observed for Carbocisteine and Internal standard.

Matrix Effect and Matrix Factor

The potential for co-extracted matrix component to influence the detector response of Analyte and Internal Standard was evaluated in six independent lots of blank K_2EDTA human normal plasma, one lot of heamolyzed plasma, one lot of lipemic plasma and one lot of heparin plasma. Aqueous standard equivalent to LQC and HQC level concentration along with intended concentration of Internal Standard were spiked to the post extracted blank matrix respectively for both LQC and HQC samples, respectively.

The matrix effect for IS normalized at LQC and HQC level were found to be between 0.93 to 1.05 and 0.99 to 1.01, respectively. The percentage CV of Matrix factor for IS normalized at LQC and HQC level were found to be 3.75% and 0.84%, respectively. The Matrix effect of Carbocisteine for LQC and HQC were found to be 101.65% and 99.72%, respectively.

The matrix factor for IS normalized at LQC and HQC level were found to be between 0.94 to 1.08 and 0.94 to 1.03, respectively. The percentage CV of Matrix factor for IS normalized at LQC and HQC level were found to be 4.83%

and 3.09%, respectively. The Matrix effect of Carbocisteine for LQC and HQC were found to be 101.76% and 98.40%, respectively.

Precision and Accuracy
Assay Precision and Accuracy (inter batch and intra batch) values were determined across three precision and accuracy batches by analyzing six replicates each of LOQQC, LQC, INTQC, MQC, and HQC samples in each batch.

Recovery of Carbocisteine
The recovery of Carbocisteine determined by comparing the detector response of Analyte at three distinct levels of extracted low, medium and high quality control samples against Precision and Accuracy with detector response obtained from unextracted aqueous quality control samples. The average recovery of Carbocisteine was 60.89%. The percentage CV for Carbocisteine recovery at three different QC level were 4.60%.

Recovery of Internal Standard
The recovery of IS determined by the average detector response of IS in extracted low, medium and high quality control samples against Precision and Accuracy samples with average detector response obtained from unextracted aqueous quality control samples. The mean recovery of Internal Standard was 68.24%.

Stability Studies
Freeze-Thaw Stability
Six replicates of Carbocisteine samples at LQC and HQC concentration in K_2EDTA human plasma were analysed after four freeze-thaw (FT4) cycles (at both -70ºC ± 15ºC and -30ºC ± 10ºC storage temperatures). The stability was determined by calculating the percentage nominal of LQC and HQC samples against freshly spiked, prepared calibration curve standards.

The mean percentage nominal of FT4 (Fourth Freeze Thaw cycle) stability samples calculated against freshly spiked, prepared CC at LQC and HQC concentrations for -70ºC ± 15ºC and -30ºC ± 10ºC were 96.14% & 101.79% and 95.39 & 104.98%, respectively demonstrating acceptable four freeze thaw stability cycles.

Wet Extract Stability
Wet Extract Stability in Refrigerator
Six replicates of LQC and HQC were processed and reconstituted with reconstituted solution. The stability samples were transferred into injector vials and stored for 76.08 hours in refrigerator at 2-8°C. Stored stability samples were transferred to auto sampler and analysed against freshly spiked, prepared calibration curve standards.

The mean percentage nominal of wet extract stability in refrigerator samples calculated against freshly prepared CC at LQC and HQC were 91.88% & 99.34%, respectively demonstrating acceptable wet extract stability for 76.08 hours in refrigerator.

Wet Extract Stability at Room temperature
Six replicates of LQC and HQC were processed and reconstituted with reconstitution solution. The stability samples were transferred into injector vials and stored for 8.75 hours on bench at room temperature. The stability samples were injected and analyzed against freshly prepared spiked, calibration curve standards.

The mean percentage nominal of wet extract stability in room temperature samples calculated against freshly prepared CC at LQC and HQC were 94.30% & 102.20%, respectively demonstrating acceptable wet extract stability for at least 8.75 hours at room temperature.

Bench-Top Stability
Bench top stability of Carbocisteine in K_2EDTA human plasma were evaluated at room temperature. Six replicates of LQC and HQC samples were processed after keeping the samples on bench for about 10.25 hours. Bench top stability were assessed by calculating percent nominal at LQC and HQC level against freshly spiked, prepared calibration curve.

The mean percentage nominal of bench top stability samples calculated against freshly prepared CC at LQC and

HQC levels were 93.23% & 96.88%, respectively demonstrating acceptable bench-top stability for at least 10.25 hours at room temperature.

Auto Sampler Stability for Carbocisteine
Six replicates of LQC and HQC samples processed for Precision and accuracy were retained after completing the batch analysis and kept stored in auto sampler at 15°C for 77.57 hours. The auto sampler stability was determined against freshly spiked, prepared calibration curve standards.

The mean percentage nominal of auto sampler stability samples calculated against freshly prepared CC at LQC and HQC levels after 77.57 hours at 15°C were 93.63% & 100.69%, respectively demonstrating acceptable auto sampler stability for at least 77.57 hours at 15°C.

Auto Sampler Stability for Internal Standard
Six replicates of LQC and HQC samples processed for Precision and Accuracy were retained after completing the batch analysis and kept stored in auto sampler at 15°C for 77.57 hours.The auto sampler stability of internal standard determined by comparing the Internal Standard area of stability samples against internal standard area of comparison samples at LQC and HQC levels. The percentage of auto sampler stability samples when compared with comparison samples at LQC and HQC levels were 99.59%, demonstrating acceptable auto sampler stability for at least 77.57 hours at 15°C.

Short-Term Stock Solution Stability for Carbocisteine
The stock solution of 1061.8056 µg/mL of the Carbocisteine was divided in two parts. One portion were diluted at two levels of low and high (LQC and HQC) concentration, placed on the bench at room temperature for 45.50 hours and other portion in refrigerator until analysis. The stability of the Carbocisteine stock solution placed on the bench at room temperature for 45.50 hours (Low and high) were compared against the freshly prepared stock dilutions at LQC and HQC level from other portion stored in the refrigerator. The percentage stability of Analyte for LQC and HQC were 100.98% & 99.39%, respectively.

Short-Term Stock Solution Stability for Internal Standard
The stock solution of Internal Standard with concentration 1027.4928 µg/mL was divided in two portions. One portion were diluted to intended internal standard concentration (25.0023µg/mL) and placed on the bench top at room temperature for 45.50 hours and other portion in refrigerator until analysis. The stability of the internal standard stock solution placed on the bench at room temperature for 45.50 hours were compared against the freshly prepared internal standard concentration from other portion stored in the refrigerator. The percentage stability of Internal Standard for LQC and HQC were 99.61% & 100.47%, respectively.

Long Term Stock Solution Stability for Carbocisteine
Stock solution 1003.9432 µg/mL of Carbocisteine were kept in the refrigerator for 19 days.A fresh stock of 1061.8056µg/mL were prepared on the day of analysis. Both stocks were diluted to LQC and HQC equivalent concentration of 7.4211µg/mL & 7.4029µg/mL and 265.0410 µg/mL & 264.3896 µg/mL for stored and fresh stock, respectively.The area ratio of stability stock solution were compared against freshly prepared stock solution. The % stability of Carbocisteine were 99.84% and 99.93%, demonstrating stock solution stability for atleast 19 days.

Long Term Stock Solution Stability for Internal Standard
The stock solution of 1015.2315 µg/mL of Internal Standard were kept in the refrigerator for 19 days. A fresh stock of 1027.4928 µg/mL were prepared on the day of analysis. Both stocks were diluted to IS dilution equivalent concentration of 25.0424 µg/mL and 25.0023 µg/mL for stored and fresh stock respectively. The area ratio of stability stock solution were compared against freshly prepared stock solution. The % stability of Internal Standard were 98.57% and 99.72%, demonstrating stock solution stability for atleast 19 days.

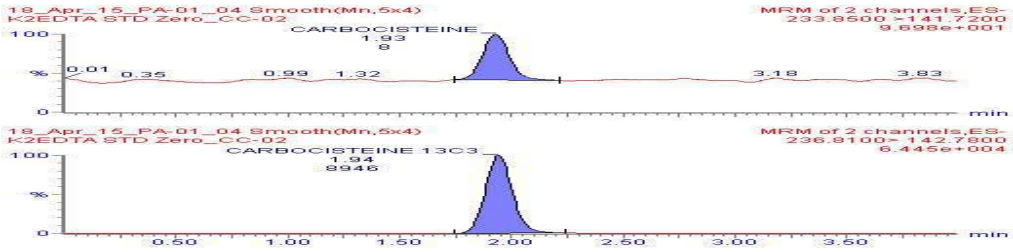

Figure 6: Representative Chromatogram of a Standard Zero for Carbocisteine

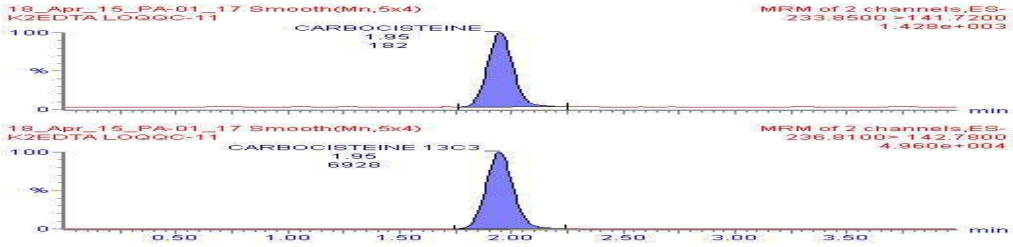

Figure 7: Representative Chromatogram of LOQQC Sample for Carbocisteine

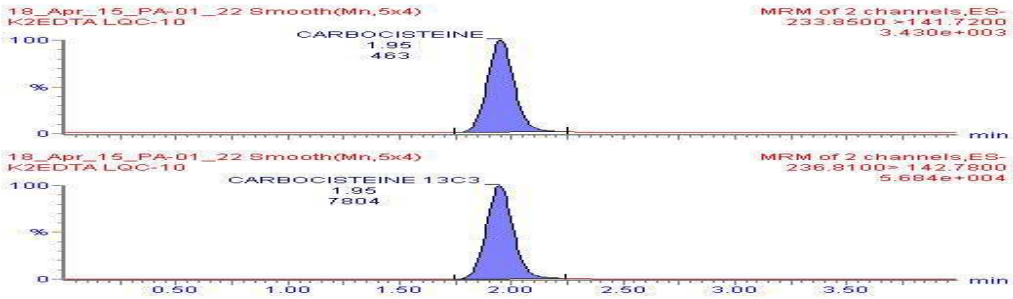

Figure 8: Representative Chromatogram of LQC Sample for Carbocisteine

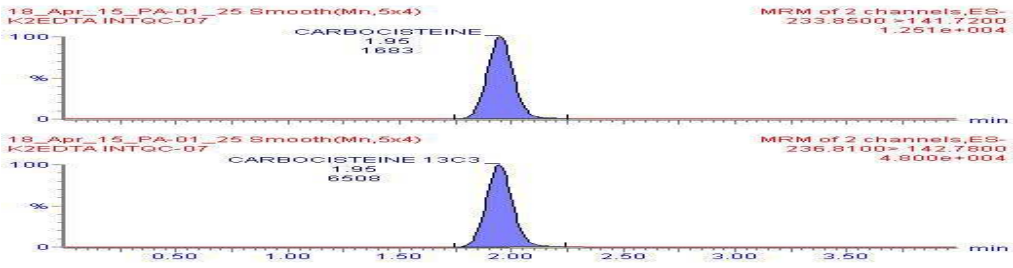

Figure 9: Representative Chromatogram of INTQC Sample for Carbocisteine

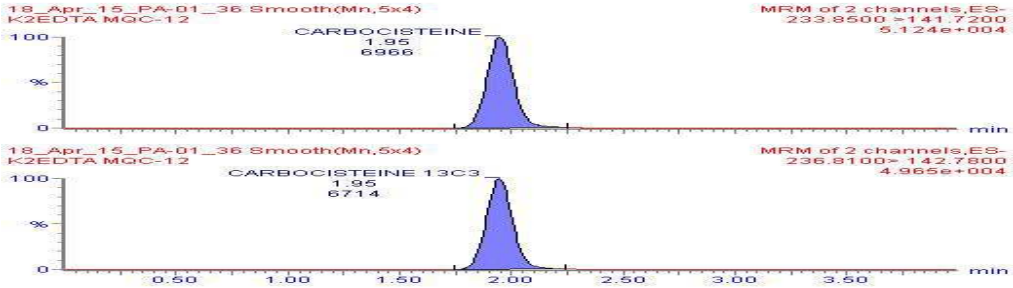

Figure 10: Representative Chromatogram of MQC Sample for Carbocisteine

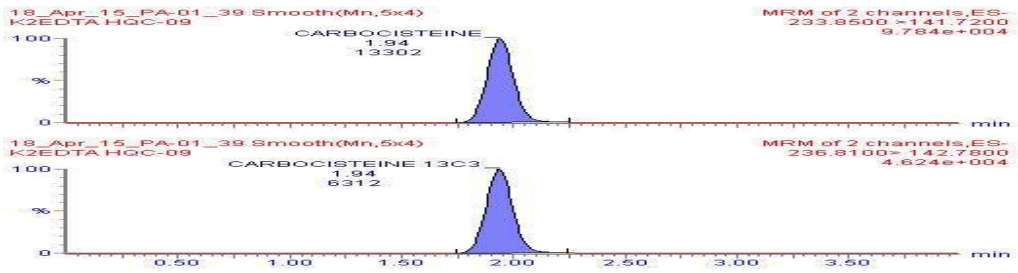

Figure 11: Representative Chromatogram of HQC Sample for Carbocisteine

Pharmacokinetic Application

This validated method was applied for an open label, balanced, randomized, two-treatment, two period, two sequence, single dose, two-way crossover bioequivalence study comparing a carbocisteine 375 mg capsule in 58 healthy, adult, human subjects under fasting conditions. In each period, a total of 18 blood samples (5 mL each) were collected. In the blood sampling, the first sample was collected within 75 minutes prior to drug administration (0.00 hour) and subsequent samples were collected at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.33, 2.67, 3.00, 4.00, 5.00, 6.00, 8.00, 10.00, 12.00 h after drug administration. All the samples were centrifuged in a refrigerated centrifuge at 2°C to 8°C with a rate of 3500 rpm for 10 min, as soon as possible to separate the plasma. The statistical analysis was performed on the pharmacokinetic parameters of the carbocisteine capsule using SAS®, statistical software Version 9.2; SAS Institute Inc., USA. The study was initiated after the appropriate ethics committee's approval of the protocol and the informed consent documents.

The primary objective for this study was to demonstrate the bioequivalence between two carbocisteine formulations in 58 healthy, adult, human subjects under fasting conditions on the basis of the pharmacokinetic (PK) parameters: C_{max} and AUC_{0-t}. The secondary objective was to monitor the safety of subjects and to assess other pharmacokinetic data on the basis of PK parameters: Tmax, t1/2, K_{el} and $AUC_{0-\infty}$.

The subjects were housed in the clinical facility from at least 11.00 hours pre-dose to 12.00 hours post dose in each period. The subjects have stayed for 1 day night and 1 day in the facility in each period. There was a washout period of at least 5 days between the successive dosing day and study duration was for 7 days. The subjects were served with dinner on day of check-in of each period. Thereafter subjects were fasted for at least 08.00 hours pre-dose to 04.00hours post dose.Standard meals was provided after 04.00 hours of drug administration at appropriate time intervals (at around 04.00 and 09.00hours post dose). Water was restricted at least 01.00 hour prior to dosing until 01.00 post dose (Except for water given during dosing). Free access to water was allowed after 01.00 hour post dose. Subjects were instructed to abstain from consuming caffeine and/or xanthenes containing products (i.e. coffee, tea, chocolate and caffeine containing- sodas, colas etc.) for at least 24.00 hours prior to check in and throughout the study grapefruit and/or its juice and poppy containing foods for at least 48.00 hours prior to check in and throughout the study.

Studies in humans have demonstrated that carbocisteine reduces goblet cell hyperplasia. Carbocisteine can therefore be demonstrated to have a role in the management of disorders characterized by abnormal mucus. Carbocisteine is rapidly absorbed from the GI tract. In an in-house study, at steady state (7 days) carbocisteine capsules 375mg given as 2 capsules t.d.s to healthy volunteers gave the pharmacokinetic parameters as given in table 3 and table 4.

Table 3: Pharmacokinetic parameters for carbocisteine

Plasma Determinations	Mean	Range
T_{Max} (Hr)	2.0	1.0-3.0
C_{Max} (ng/ml)	5809.40	-
$T_{1/2}$	1.87	1.4-2.5
K_{el} (Hr^{-1})	0.387	0.28-0.50
$AUC_{0-7.5}$(mcg.Hr.ml^{-1})	39.26	26.0-62.4

Table 4: Derived Pharmacokinetic parameters for carbocisteine

Derived Pharmacokinetic parameters	Observed values
*$CL_s(L.Hr^{-1})$	20.2
$CL_s(ml.min^{-1})$	331
$V_D(L)$	105.2
$V_D(L.Kg^{-1})$	1/75

*****Calculated from dose for day 7 of study.*

CONCLUSION

This analytical method is valid for determination of Carbocisteine (over a range of 52.27 µg/ml to 5301.83 µg/ml) using as Carbocisteine 13 C 3 internal standard in human plasma. Signal from the detector were captured in a computer and processed using MassLynx SCN 4.1 v software. This method for quantification of carbocisteine in human plasma is accurate, precise, rapid, and selective. It is a simple, practical, and economical alternative for studies of the bioavailability, bioequivalence, and pharmacokinetics of this drug in human plasma.

Acknowledgement
I avail this opportunity, with great pleasure and deep sense of gratitude, to express my thanks to my research fellows and especially Mr. Sunil Kumar for providing enough patience, zeal and strength that enabled me to complete this work successfully.

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