

High-throughput determination of carbocysteine in human plasma by liquid chromatography/tandem mass spectrometry: application to a bioequivalence study of two formulations in healthy volunteers

Hui-chang Bi¹, Li-zi Zhao¹, Guo-ping Zhong¹, Shufeng Zhou², Bo Li¹, Ying Deng¹, Xiao Chen³ and Min Huang^{1*}

¹Institute of Clinical Pharmacology, School of Pharmaceutical Sciences, Sun Yat-sen University, 74 Zhongshan Road II, Guangzhou 510080, China

²Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore

³Department of Pharmacy, the First Affiliated Hospital, Sun Yat-sen University, 58 Zhongshan Road II, Guangzhou 510080, China

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A rapid and sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method to determine carbocysteine in human plasma was developed and fully validated. After methanol-induced protein precipitation of the plasma samples, carbocysteine was subjected to LC/MS/MS analysis using electrospray ionization (ESI). The MS system was operated in the selected ion monitoring (SRM) mode. Chromatographic separation was performed on a Hypurity C₁₈ column (i.d. 2.1 mm × 50 mm, particle size 5 μm). The method had a chromatographic running time of 2.0 min and linear calibration curves over the concentration ranges of 0.1–20 μg/mL for carbocysteine. The lower limit of quantification (LLOQ) of the method was 0.1 μg/mL for carbocysteine. The intra- and inter-day precision was less than 7% for all quality control samples at concentrations of 0.5, 2.0, and 10.0 μg/mL. These results indicate that the method was efficient with a simple preparation procedure and a very short running time (2.0 min) for carbocysteine compared with methods reported in the literature and had high selectivity, acceptable accuracy, precision and sensitivity. The validated LC/MS/MS method has been successfully used to a bioequivalence study of two tablet formulations of carbocysteine in healthy volunteers. Copyright © 2006 John Wiley & Sons, Ltd.

Carbocysteine, *S*-carboxymethyl-L-cysteine (for chemical structure, see Fig. 1), a dibasic amino acid, is a mucoregulating agent.¹ It diminishes the viscosity and increases the volume of pathologically thickened sputum thereby facilitating expectoration.¹ In clinical practice, carbocysteine has gained acceptance and is used in the management of respiratory diseases characterized by accumulation of excessive secretions.^{1–3}

A crucial aspect in the determination of the pharmacokinetic parameters of any pharmaceutical preparation is the quantification of the compound in human plasma. To determine this drug in human plasma, a rapid, accurate and reliable analytical method is needed. For the lack of absorption in the visible and UV ranges, various analytical methods reported for the measurement of carbocysteine in biomatrix rely on the detection of UV-active, fluorescent functional or volatile derivatives. These methods include gas chromatographic methods with prior derivatization with

heptafluorobutyric anhydride or acetic anhydride,^{4,5} ion-exchange chromatography following derivatization prior to UV detection,^{6,7} high-performance liquid chromatography (HPLC) with pre-column derivatization (with phenyl isothiocyanate,^{8,9} *o*-phthalaldehyde,^{10–12} dabsyl chloride,^{13,14} and 9-fluorenylmethyl chloroformate using fluorescent pre-column labeling¹⁵), and liquid chromatography/tandem mass spectrometry (LC/MS/MS) with atmospheric-pressure chemical ionization (APCI) employing precolumn derivatization.¹⁶ These methods often suffered from a very complicated and time-consuming derivatization procedure before analysis. At the same time, gradient elution or a column-switching technique had to be used to avoid possible interference from endogenous amino acids, which may have chemical properties similar to carbocysteine. Recently, another liquid chromatographic/mass spectrometric method with electrospray ionization (ESI) and in the selected-ion monitoring mode has been used to determine carbocysteine in human plasma without any derivatization step.¹⁷ However, this method required a long chromatographic separation time (>16 min) to eliminate possible endogenous interference,¹⁷ which is not suitable for high-throughput analysis when a large number of samples need to be quantitated.

*Correspondence to: M. Huang, Institute of Clinical Pharmacology, School of Pharmaceutical Sciences, Sun Yat-sen University, 74 Zhongshan Road II, Guangzhou 510080, China.

E-mail: huangm@gzsums.edu.cn

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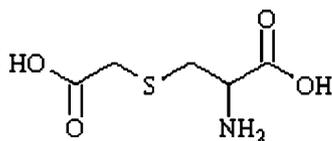


Figure 1. Chemical structure of carbocysteine.

To overcome all these problems, we developed a rapid and sensitive LC/MS/MS method to measure carbocysteine in human plasma using a one-step protein precipitation without the need for derivatization. The method exhibited excellent performance in terms of selectivity, robustness, and excellent efficiency (2.0 min of running time per sample) with simplicity of sample preparation. The method was fully validated and successfully applied to a bioequivalence study of two tablet formulations of carbocysteine in 20 healthy Chinese volunteers.

EXPERIMENTAL

Chemicals and reagents

Carbocysteine was provided by Yichang Sanxia Pharmaceutical Co. (Wuhan, China). This had a relative purity of 100.1% as compared with the standards from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and formic acid of HPLC grade were all purchased from Tedia Company Inc. (Fairfield, OH, USA). All other reagents were of analytical grade. Blank human plasma was obtained from Guangzhou Blood Center (Guangzhou, China). Ultra-pure water was obtained from a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA).

Preparation of standard and quality control samples

The stock solution of carbocysteine was prepared by dissolving the accurately weighed reference compound in hot water (70–80°C) to give a final concentration of 200 µg/mL. The solutions were then serially diluted with methanol/water (10:90, v/v) to obtain standard working solutions at concentrations of 1.0, 5.0, 10.0, 20.0, 50.0, and 100.0 µg/mL. All the solutions were stored at 4°C and were brought to room temperature before use. Calibration standard solutions were prepared by spiking blank human plasma with carbocysteine standard solutions to give concentrations of 0.1, 0.25, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 µg/mL. Quality control (QC) samples, which were used both in prestudy validation and during each experimental run of the bioequivalence study, were prepared by spiking control human plasma with carbocysteine standard solutions to give concentrations of 0.5, 2.0, and 10.0 µg/mL for carbocysteine.

Sample preparation

To 200 µL human plasma in a 1.5-mL test tube, 400 µL of methanol were added. After vortexing for 10 s and centrifugation at 3000 g for 4 min, an aliquot of 5 µL of the

clear supernatant was directly injected onto the LC/MS/MS system for analysis.

Liquid chromatographic and mass spectrometric conditions

A Finnigan Surveyor MS pump (San Jose, CA, USA) and a Finnigan Surveyor autosampler were used for solvent and sample delivery. Chromatographic separation was achieved by using a Hypurity C₁₈ column (i.d. 2.1 mm × 50 mm, 5 µm; Thermo Electron Corporation, USA) at 30°C. The mobile phase consisted of methanol/water (containing 0.1% formic acid) (50:50, v/v), pumped at a flow rate of 200 µL/min. Total running time was 2.0 min for each injection.

A Finnigan TSQ Quantum triple quadrupole mass spectrometer equipped with an ESI source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive-ion mode (ESI+) and set up in the selected reaction monitoring (SRM) mode. Nitrogen was used as the sheath gas (45 psi) and the auxiliary gas (1 psi). The capillary temperature was 350°C. The spray voltage was 3500 V. Collision-induced dissociation (CID) studies were performed and argon was used as the collision gas with a collision cell gas pressure of 1.2 mTorr (1 Torr = 133.3 Pa). The collision energy was 12 eV for carbocysteine. Based on the full-scan mass spectra of the drug, the most abundant ions were selected and the mass spectrometer was set to monitor the transitions of the precursors to the product ions as m/z 180 → 89 for carbocysteine. Q1 and Q3 peak widths (FWHM) were set to 0.5 and 0.7 amu, respectively. The scan time for each analyte was set to 0.1 s. Full-scan mass spectra of $[M+H]^+$ of carbocysteine and the respective product-ion spectra are shown in Fig. 2. Data acquisition was performed with Finnigan Xcalibur 1.3 software. Peak integration and calibration were performed with Finnigan Lcquan software.

Method validation

The method was validated for selectivity, accuracy, precision, recovery, calibration curve range, and reproducibility according to the FDA guideline for validation of bioanalytical methods.¹⁸ The selectivity was investigated by preparing and analyzing six individual human blank plasma samples at the LLOQ. The LLOQ was defined as the lowest concentration of the analyte measured with acceptable precision and accuracy [relative standard deviation (RSD) and relative error <20%], and the analyte's response at this concentration level was >5 times of the baseline noise. Linearity was assessed by analyzing carbocysteine standards (0.1–20 µg/mL) in human plasma. Calibration curves were analyzed by weighted linear regression (1/ x) of assayed-nominal drug peak area ratios.

Accuracy and precision were assessed by determining QC samples at three concentration levels (five samples each concentration) on three different validation days. The precisions were determined as the RSD (%) and the accuracies were expressed as a percentage of the nominal concentration. The criteria used to assess the suitability of precision and accuracy was as follows: the RSD should not exceed 15% and the accuracy should be within 80–120%. Furthermore, the recovery (extraction efficiency) of analyte from human plasma was determined by comparing the areas

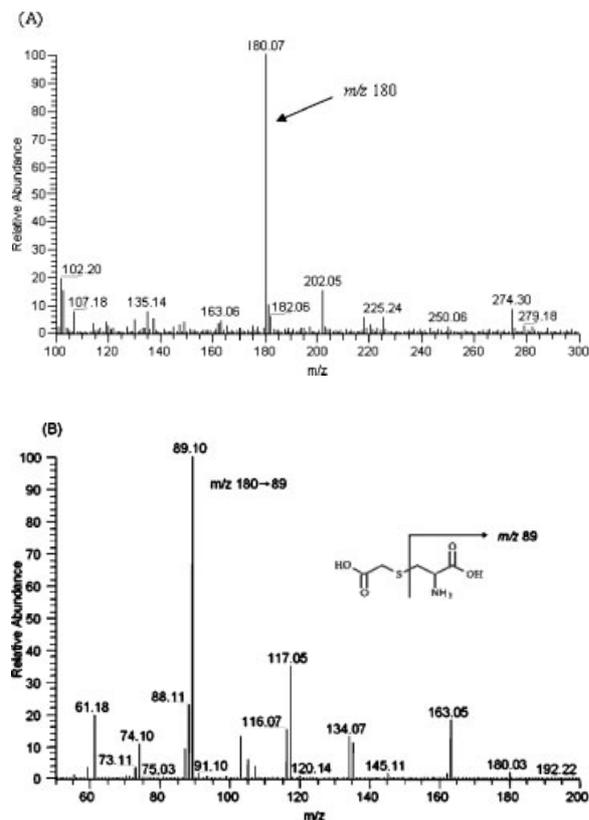


Figure 2. (A) Mass spectrum of carbocysteine in the positive ion mode ($[M+H]^+$) using ESI and (B) MS/MS product-ion spectrum of carbocysteine with $[M+H]^+$ at m/z 180 as the precursor ion.

of spiked plasma samples before and after liquid extraction that represent 100% recovery.

The stability of an analyte was assessed by determining QC samples at three concentrations (five samples each), exposed to different time and temperature conditions. The stability studies included: (a) stability at room temperature for 4 h; (b) stability after three freeze/thaw cycles; (c) stability of the extracted samples at room temperature for 12 h; and (d) the long-term stability after storage at -30°C for 23 days. During routine analysis, each analytical run included blank plasma, a set of calibration samples, a set of QC samples and unknowns.

Bioequivalence study

The validated method was applied to a bioequivalence study of dispersible tablets containing 100 mg and 250 mg carbocysteine. Twenty healthy adult male volunteers participated in this study. The volunteers were all healthy adult Chinese who were selected after a thorough medical, biochemical and physical examination. Informed consent was obtained from all the subjects after explaining the aim and risks of the study. The study protocol was approved by the Human Investigation Ethical Committee of the School of Pharmaceutical Sciences at the Sun Yat-sen University, Guangzhou, China.

The study was of a single-dose, randomized, two-period crossover design. Dosing periods were separated by a 1-week washout period. After an overnight fasting (10 h), the volunteers took the assigned tablet orally with 200 mL of

water. Regular standardized low-fat meals were provided at 4 h after dose administration, and water intake was allowed at 2 h following drug administration. Following drug administration, venous blood samples (3.0 mL) were collected into heparinized tubes at the following times: immediately before administration, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, and 10 h after dosing. Blood samples were centrifuged at $1500g$ for 10 min to obtain the plasma. The plasma samples were labeled and kept frozen at -30°C until analysis.

Calculation of pharmacokinetic parameters was done using the Nonmem program (version 1.1). The elimination rate constant (β) was obtained as the slope of the linear regression of the log-transformed concentration values versus time data in the terminal phase. The elimination half-life ($t_{1/2\beta}$) was calculated as $0.693/\beta$. Time to peak plasma concentration (T_{max}) and peak plasma concentration (C_{max}) were read directly from the observed concentration versus time profiles. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve to infinity ($\text{AUC}_{0-\infty}$) was calculated as $\text{AUC}_{0-t} + C_t/\beta$, where C_t is the last measurable concentration.

For the bioequivalence analysis, C_{max} , AUC_{0-t} and $\text{AUC}_{0-\infty}$ were considered as primary variables. Statistical significance of variations in the different formulations was tested according to a one-way analysis of variance (ANOVA) followed by Dunnett's test using the Excel 2000 program. The products were considered bioequivalent if the difference between the two compared parameters was statistically insignificant ($P \geq 0.05$) and 90% confidence interval (CI) for these parameters fell within 80–125%.

RESULTS AND DISCUSSION

Method development

In the analysis of the target compound, ESI was chosen as the ionization source. The signal intensity of carbocysteine in human plasma was high using the ESI source and this source provided satisfactory data on method validation and subsequent quantitation for plasma samples from healthy volunteers. By ESI, carbocysteine formed predominantly protonated molecules $[M+H]^+$ of m/z 180 in full-scan spectra. To determine carbocysteine using SRM mode, full-scan and product-ion spectra of the analyte were investigated. The most abundant ion in the product-ion mass spectrum was found at m/z 89 for carbocysteine. It was found that the capillary temperature and the spray voltage did not significantly influence the MS behavior of the analyte and remained unchanged at the recommended value of 350°C and 3.5 kV. Other MS conditions, including source CID and collision pressure, were maintained at the auto-tuned value since they did not significantly affect the spectral behavior of the analyte. However, the collision behavior of the $[M+H]^+$ of the analyte was strongly dependent on the collision energy. An increase in the collision energy caused a marked increase in the fragmentation processes. A fragment ion at m/z 89 was formed using 5 eV collision energy. When higher collision energy was used, the relative abundance of this ion increased and reached the maximum value using 12 eV. The

Table 1. Effect of collision energy on the fragment ion formation of carbocysteine in product-ion scan mode

Collision energy (eV)	Fragment ions (<i>m/z</i> (relative abundance))
5	89(16), 117(11), 134(22), 163(100)
10	89(96), 117(45), 134(37), 163(100)
12	89(100), 117(37), 134(16), 163(26), 61(18)
15	89(100), 117(35), 134(12), 163(18), 61(20)

formation of fragment ions of carbocysteine under variable collision energy is presented in Table 1. After optimization of the collision energy, the collision behavior was investigated using 12 eV collision energy for carbocysteine and the SRM transition of m/z 180 \rightarrow 89 for carbocysteine was selected to obtain maximum sensitivity. Positive-ion ESI mass spectrum for the target compound and MS/MS product-ion spectrum of the compound are shown in Fig. 2.

In the present study, a simple single-step liquid-liquid protein precipitation procedure was used. Different kinds of extraction procedures, including liquid-liquid extraction (LLE) using dichloromethane, chloroform, ethyl acetate, and solid-phase extraction (SPE) carried out on HLB Oasis and MCX Oasis cartridges (Waters Corp., Milford, MA, USA), were investigated and compared during our method development. The results were depressed with bad peak shape, low extraction efficiency, very low response, or even not detectable. However, the response was increased when the protein precipitation procedure was used. The extraction efficiency and matrix effect (ME) of different protein precipitants including acetonitrile, alcohol, and methanol were compared during our method development. All solvents gave very low (about 10%) extraction efficiency for carbocysteine, which was very similar to that reported in the literature.¹⁷ The extraction recovery of this reported method using acetonitrile-induced protein precipitation was about 20%, and it was pointed out that most of the compound was lost during protein precipitation.¹⁷ In our study, acetonitrile as a precipitant had similar extraction efficiency to methanol, but it increased the ME and the peak shape was worse than that when methanol was used. Methanol also showed a ME and decreased the mass spectral response to the analytes, but to a lesser extent compared to acetonitrile. However, methanol caused a lower ME and insignificant effect on spectral response to the analyte with a better peak shape compared to other organic solvents. Thus, methanol was finally used as the protein precipitant throughout the study.

Various combinations of methanol and water with changed content of each component were investigated and compared to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. The acidic modifier, formic acid, in the mobile phase and an increase in the water content could improve peak shape, whereas an increase in the methanol content worsened the peak. Lower acid concentration (water containing 0.1% formic acid instead of 1.0% formic acid) was better for ionization efficiency. A mobile phase consisting of methanol/water (containing 0.1% formic acid) was finally used and the ratio of 50:50 (v/v) was optimal. Each chromatographic run was completed within 2.0 min.

Usually, an internal calibration using a stable isotopic or a chemical analog internal standard (IS) is more robust than an external calibration using absolute peak area in the case of there are no mutual cross-talk and ion-suppression phenomena between the analyte and the IS. An ideal IS should be a structurally similar analog, stable isotope labeled compound according to the FDA guideline.¹⁸ However, it was difficult to find a commercially available IS that has similar chemical and physical behavior to carbocysteine when we started the method development. Huperzine A, an alkaloid isolated from the Chinese herb *Huperzia serrata*,¹⁹ was tried as the IS at the beginning of method development, which could be readily extracted using the current processing method and had similar chromatographic behavior under the present LC/MS/MS conditions. Unfortunately, very strong ion suppression on carbocysteine caused by huperzine A was observed during the method validation. On the other hand, when an external calibration using absolute peak area was performed, the results of method validation were acceptable in this study based on the FDA guideline.¹⁸ Furthermore, to assure that the absolute peak response was reliable, the calibrations and QC samples were run before and after a series of unknown samples to monitor whether the instrument performance decreased over time. Fortunately, the instrument performance was stable during our analytical period. Taking all these together, the current external calibration method was reliable and suitable for this bioequivalence study.

Method validation

Selectivity

The LC/MS/MS method demonstrated high specificity because only ions derived from the analytes of interest were monitored. The selectivity towards endogenous plasma matrix was tested in six different batches of human plasma samples by analyzing blanks and samples at LLOQ levels. Observing the chromatographs indicated no significant visible interference at the expected retention times of the analyte since carbocysteine was modified to elute in a region where visible interference is not observed. Chromatograms of blank human plasma and the plasma at LLOQ levels are shown in Fig. 3. The retention time for carbocysteine was 0.95 min. The method had the shortest total running time (2.0 min) for determination of carbocysteine in human plasma compared with those reported in the literature.^{4–17}

Matrix effects

Matrix effect (ME) occurs when a biological sample contains a component that does not give a signal in the SRM channel used for the target analyte but co-elutes with the analyte and affects (usually attenuates) the response of the analyte.²⁰ The presence of a ME can decrease or increase the response of the analyte and thus affect sensitivity of the method. Therefore, the assessment of the ME is critical for the reliable evaluation of newly developed LC/MS/MS methods, in particular when external calibration instead of internal calibration is utilized in the methods.

The absolute and relative ME on the spectral response of carbocysteine was assessed as described by Matuszewski

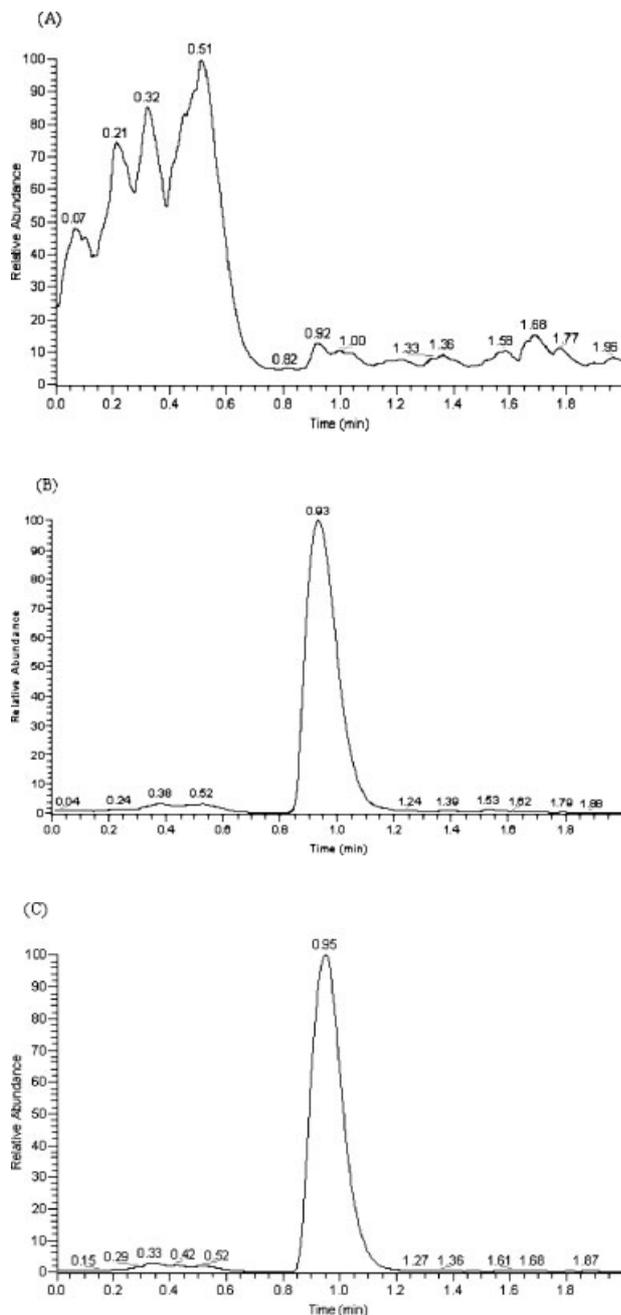


Figure 3. Representative selected reaction monitoring (SRM) chromatograms of carbocysteine in human plasma: (A) a blank plasma sample; (B) a blank plasma sample spiked with carbocysteine at the LLOQ of 0.1 $\mu\text{g}/\text{mL}$; and (C) plasma sample from a healthy volunteer 10 h after an oral administration of 1000 mg carbocysteine.

et al.,²¹ which we slightly modified and simplified as mentioned previously.²² To evaluate the absolute ME, i.e. the potential ion suppression or enhancement due to the matrix components, five different batches of blank plasma were extracted by methanol and then spiked with the analyte at QC concentrations. The corresponding peak areas of the analyte in spiked plasma post-extraction (B) were then compared with those of the aqueous standards in mobile phase (A) at equivalent concentrations. The ratio $(B/A \times 100)$ is defined as the ME. A ME value of 100% indicates that the response in the mobile phase and in the plasma extracts was

the same and no absolute matrix effect was observed. A value of $>100\%$ indicates ionization enhancement, and a value of $<100\%$ indicates ionization suppression.

The result of ME at QC concentrations of carbocysteine in five different lots of human plasma shows that there was ME as indicated by values of $<100\%$ (range from 81.2–85.1%) in the area of the analyte in spiked plasma samples post-extraction. This indicated that there was an ionization suppression for carbocysteine under the present chromatographic and extraction conditions when an ESI interface was employed. Fortunately, the ionization suppression observed was similar and kept consistent over the QC concentration ranges of the analyte (0.5–10.0 $\mu\text{g}/\text{mL}$) without showing any analyte concentration-dependence as well as for different lots of human plasma. Moreover, such ionization did not affect the slopes (215047.5 ± 7233.2) and linearity ($r^2 > 0.998$) of the established calibration curves over the whole analytical period.

The assessment of the relative ME was made by a direct comparison of the analyte peak area values between different lots (sources) of plasma. The variability in the values, expressed as RSD (%), is a measure of the relative ME for the target analyte. The variability was acceptable with RSD values $<7.8\%$ at different concentrations of carbocysteine in five different lots of human plasma, indicating that the relative ME for the analyte was minimal in this study.

In the present study, an ionization suppression effect due to the undetected matrix components in human plasma was observed. However, such ionization suppression kept consistent over the QC concentration ranges of the analyte without showing any analyte concentration-dependence and did not significantly affect the behaviors of calibrations curves, precision and accuracy data. Thus, despite the presence of the ME, the present analytical method was reliable.

Linearity and lower limit of quantification

The slope, the intercept, and the correlation coefficient (r) for each standard curve from each analytical run were determined automatically by Finnigan Lcquan software program. Table 2 shows the mean slope, intercept and correlation coefficient values for carbocysteine. The mean squared correlation coefficients (r^2) for the daily calibration curves were all ≥ 0.998 ($n=5$) for carbocysteine and the within- and between-run coefficients of variance (CVs) of the response factors for each concentration assayed were $<10\%$. The mean slope and intercept were 215047.5 ± 7233.2 and 1786.5 ± 193.7 with RSDs of 3.4% and 10.8%, respectively. For each point on the calibration curves for the analyte, the concentrations back-calculated from the equation of the regression analysis were within acceptable limits for accuracy and precision of $\pm 10\%$. Overall, carbocysteine

Table 2. Summary of calibration curve statistics for the studied drug ($n=5$)

Conc. range of carbocysteine ($\mu\text{g}/\text{mL}$)	Slope		Intercept		r^2
	Mean \pm SD	RSD (%)	Mean \pm SD	RSD (%)	
0.1–20.0	215047.5 ± 7233.2	3.4	1786.5 ± 193.7	10.8	>0.998

gave a linear response as a function of the concentration ranges studied and showed excellent linearity over the range 0.1–20.0 µg/mL (Table 2).

The lowest concentration on the calibration curve of carbocysteine was 0.1 µg/mL. The analytes' response at these concentration levels were >20 times of the baseline noise. The precision and accuracy at these concentration levels were acceptable, with <10.4% of the CVs. Thus, the lowest concentration on the calibration curve was accepted as the LLOQ. Based on a simple calculation, about 0.016 ng was injected on-column for a 0.1 µg/mL sample after processing, indicating that the absolute sensitivity at the LLOQ level was about 0.016 ng. The limit of detection (LOD) could be achieved at 0.02 µg/mL with spectral response about 5 times of the baseline noise. To properly evaluate pharmacokinetics of a drug, the analytical method needs to be able to measure 3–5 half-lives and 1/10 to 1/20 of C_{max} according to China SFDA and FDA guidelines.^{26,27} In the present bioequivalence study, the half-life of carbocysteine was about 1.5 h, and mean C_{max} values for both formulations ranged from 5.6–5.8 µg/mL with a last measurable concentration range of 0.13–0.47 µg/mL at 10 h after an oral dose of 1000 mg of carbocysteine. Using this analytical method with a LLOQ of 0.1 µg/mL, we were able to measure concentrations of carbocysteine in plasma samples collected up to 10 h (last time point for plasma sample collection) from all subjects after oral administration of 1000 mg of carbocysteine. Though the LLOQ could be lower and near to the LOD, we set the LLOQ at a 0.1 µg/mL level since the current LLOQ was quite sufficient for the determination of the bioequivalence of carbocysteine following a single-dose administration of the test or reference formulation in healthy volunteers.

Precision and accuracy

The intra-day and inter-day precision and accuracy data for carbocysteine are summarized in Table 3. All values of accuracy and precision were within recommended limits.¹⁸ Intra-day precision ranged between 3.0–4.3%, and the inter-day precision was between 5.1–7.0%. The mean intra-day error was between –4.1% and 0.4%, and the mean inter-day error was between –3.6% and 2.7%.

Table 3. Intraday and interday precision and accuracy data for assays of carbocysteine in human plasma ($n=5$)

Nominal conc. of carbocysteine (µg/mL)	Precision		Accuracy
	Mean ± SD	RSD ^a (%)	Mean relative error ^b (%)
Intraday			
0.5	0.5 ± 0.0	3.0	0.4
2.0	2.0 ± 0.1	3.5	–1.3
10.0	9.6 ± 0.4	4.3	–4.1
Interday			
0.5	0.5 ± 0.0	5.1	2.7
2.0	1.9 ± 0.1	7.0	–3.6
10.0	9.8 ± 0.5	5.3	–1.8

^a RSD = relative standard deviation.

^b Mean relative error = $\frac{\text{Overall mean assayed concentration} - \text{added concentration}}{\text{Added concentration}} \times 100$.

Table 4. The recovery (extraction efficiency) for carbocysteine in human plasma ($n=5$)

Nominal conc. of carbocysteine (µg/mL)	Recovery ^a (Mean ± SD, %)	RSD ^b (%)
0.5	10.5 ± 0.9	8.6
2.0	11.6 ± 0.7	6.1
10.0	11.8 ± 0.9	7.6

^a The recovery (extraction efficiency) of the analytes from human plasma was determined as the ratio of the peak area of the analyte spiked before extraction to the peak area of the analyte spiked post-extraction multiplied by 100.

^b RSD = relative standard deviation.

Recovery

Table 4 shows the recovery (extraction efficiency) of carbocysteine from human plasma following methanol extraction. The recoveries of carbocysteine from human plasma ranged from 10.5–11.8% and were similar at all analyte concentrations, which indicated that the extraction efficiency for carbocysteine using methanol was concentration-independent. This low (about 10%) extraction efficiency for carbocysteine, however, was comparable to that reported in the literature.¹⁷ The extraction recovery of the reported method using protein precipitation was about 20%, which was because, as the author mentioned, that most of the compound was lost during protein precipitation.¹⁷ The low extraction recovery of carbocysteine was probably due to rapid degradation, protein binding behavior, and the ME from plasma components. Using this analytical method with a LLOQ of 0.1 µg/mL and recovery of about 10%, we were able to measure concentrations of carbocysteine in plasma samples collected up to 10 h (last time point for plasma sample collection) from all subjects after an oral dose of 1000 mg of carbocysteine. The analytical method was able to measure 3–5 half-lives and 1/10 to 1/20 of C_{max} according to China SFDA and FDA guidelines.^{26,27} Taking all these into consideration, though the extraction recovery was low using methanol as protein precipitant, the current analytical method was still sensitive and sufficient for the determination of carbocysteine in a bioequivalence study at an oral dose of 1000 mg in healthy volunteers.

Table 5. Stability of carbocysteine under various storage conditions ($n=5$)

Storage condition	Nominal conc. of carbocysteine ($\mu\text{g/mL}$)	Calculated conc. ($\mu\text{g/mL}$)	
		Mean \pm SD	E_r^a (%)
-30°C/23 days	0.5	0.4 \pm 0.0	-12.5
	2.0	2.0 \pm 0.1	0.8
	10.0	9.6 \pm 0.7	-3.6
-30°C/3 freeze/thaw cycles	0.5	0.5 \pm 0.0	-2.6
	2.0	2.1 \pm 0.1	4.9
	10.0	10.6 \pm 0.7	5.7
Room temperature/4 h	0.5	0.6 \pm 0.0	14.2
	2.0	2.2 \pm 0.1	8.0
	10.0	10.9 \pm 0.6	9.5
Room temperature/12 h (extracted sample)	0.5	0.5 \pm 0.0	2.6
	2.0	1.9 \pm 0.1	-5.1
	10.0	10.0 \pm 0.3	-0.3

^aRelative error =

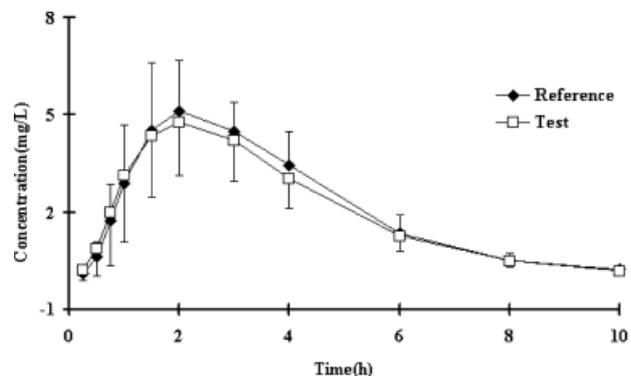
$$\frac{\text{Overall mean assayed concentration} - \text{added concentration}}{\text{Added concentration}} \times 100.$$

Stability

An analyte is considered stable in biological matrix when 85–115% of the initial concentration can be detected.¹⁸ The stability of carbocysteine in human plasma under different storage conditions is presented in Table 5. There was no significant degradation under the conditions described in this study since their concentrations deviated by no more than 14.2% relative to the reference nominal concentrations. No degradation products were detected under the selected MS conditions. Carbocysteine in human plasma can therefore be stored at room temperature for 4 h, 23 days at -30°C and after three freeze/thaw cycles. Analysis of the QC samples following the protein precipitation procedure showed no significant degradation after 12 h at room temperature. These results indicate that carbocysteine is stable under routine laboratory conditions and no specific procedure (e.g. acidification or addition of organic solvents) is needed to stabilize the compounds for daily clinical drug monitoring.

Bioequivalence study

Both test and reference formulations were well tolerated at the dose administered by all the volunteers. Unexpected incidents that could have influenced the outcome of the study did not occur. There was no drop-out and all volunteers who started the study continued to the end and the biochemical parameters remained within the reference range. Formulations used in this study were readily absorbed from the gastrointestinal tract. Carbocysteine was measurable at the first sampling time (0.25 h) in the majority of the volunteers. The mean plasma concentration-time profiles of the two formulations, shown in Fig. 4, were

**Figure 4.** Mean plasma concentration-time profile of carbocysteine after an oral dose of 1000 mg of two formulations to 20 healthy male volunteers. Each point represents the mean \pm SD.

closely similar and superimposable. ANOVA was applied to the concentrations attained at individual time intervals for both formulations, and indicated no significant difference.

The pharmacokinetic parameters of carbocysteine for the reference and test formulations are presented in Table 6. There was no significant difference between the two formulations with regard to the pharmacokinetic parameters including C_{\max} , AUC_{0-t} and $AUC_{0-\infty}$. In the current study, C_{\max} values for both formulations ranged from 5.6–5.8 $\mu\text{g/mL}$ after a single dose of 1000 mg carbocysteine, which were slightly lower than those reported in the literature.^{1,5,23,24} The values of T_{\max} obtained in the current study were in agreement with reported values.^{1,5,23,24}

From the plasma concentration versus time profiles of the 20 subjects, the relative bioavailability of carbocysteine was (94.2 \pm 13.7)% on the basis of mean $AUC_{0-\text{last}}$. The 90% CIs of carbocysteine for C_{\max} , AUC_{0-t} and $AUC_{0-\infty}$ were 88.4–105.1%, 88.3–98.5%, and 90.7–100.9%, respectively. The most important objective of a bioequivalence study is to assure the safety and efficacy of the test products. When two formulations of the same drug are equivalent in the rate and extent to which the active drug becomes available to the site of drug action, they are bioequivalent and thus considered therapeutically equivalent.²⁵ It is generally accepted that the equivalent range for basic pharmacokinetic parameters, such as C_{\max} , AUC_{0-t} and $AUC_{0-\infty}$, is 80–125%.^{26,27} The mean and standard deviations of C_{\max} , AUC_{0-t} and $AUC_{0-\infty}$ of the two formulations did not differ significantly, suggesting that the plasma profiles

Table 6. Pharmacokinetic parameters of two tablet formulations of carbocysteine (mean \pm SD, $n=20$)

Parameter	Carbocysteine	
	Reference formulation	Test formulation
C_{\max} ($\mu\text{g/mL}$)	5.8 \pm 1.3	5.6 \pm 1.4
T_{\max} (h)	2.3 \pm 0.9	2.2 \pm 0.8
$t_{1/2\beta}$ (h)	1.5 \pm 0.2	1.5 \pm 0.1
$AUC_{0-\text{last}}$ ($\mu\text{g mL}^{-1}$ h)	21.4 \pm 3.9	20.1 \pm 4.3
$AUC_{0-\infty}$ ($\mu\text{g mL}^{-1}$ h)	23.9 \pm 4.2	22.8 \pm 4.5

generated by the test formulation are comparable to those of the reference formulation. ANOVA, after log-transformation of the data, showed no statistically significant difference between the two formulations ($P > 0.05$). Furthermore, the 90% CIs for the ratios of test drug to reference drug for C_{\max} , AUC_{0-t} and $AUC_{0-\infty}$ were also within the range of 80–125%, which is the range accepted by the US and China Food and Drug Administration.^{26,27} Therefore, the two tablet formulations can be considered bioequivalent with regard to the extent and rate of absorption.

CONCLUSIONS

In this study, we investigated a newly developed LC/MS/MS method for the determination of carbocysteine in human plasma. The sample pretreatment was easy and fast using a single-step methanol-induced protein precipitation. The analyte, carbocysteine, was subject to LC/MS/MS analysis using an ESI technique with satisfactory mass spectral response generated. Detailed validation following the FDA guideline indicated that the developed method had high sensitivity, reliability, specificity and excellent efficiency with a total running time of 2.0 min per sample. The method was successfully applied to determine carbocysteine plasma concentrations in a bioequivalence study in human volunteers. As the 90% CIs for C_{\max} , AUC_{0-t} and $AUC_{0-\infty}$ for the two formulations were all within 80–125%, it was concluded that the test carbocysteine tablet formulation was bioequivalent to the reference formulation with regard to both the rate and the extent of absorption in humans.

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