



## Original article

# Quantitative determination of D<sub>4</sub>-cystine in mice using LC-MS/MS and its application to the assessment of pharmacokinetics and bioavailability

Shuning Li, Zhenyao Lu, Li Jiao, Ran Zhang, Yu Hong, Jiye Aa<sup>\*</sup>, Guangji Wang

Jiangsu Province Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing, Jiangsu, 210009, China

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## ABSTRACT

Cystine is the primary source material for the synthesis of glutathione. However, the pharmacokinetics and tissue distribution of cystine are largely unknown. A surrogate analyte D<sub>4</sub>-cystine was employed to generate calibration curves for the determination of levels of D<sub>4</sub>-cystine and endogenous cystine in mice by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Validation assessments proved the sensitivity, specificity and reproducibility of the method with a lower limit of quantification (LLOQ) of 5 ng/mL over 5–5000 ng/mL in plasma. The pharmacokinetics of D<sub>4</sub>-cystine were evaluated after administering injections and oral solutions, both of which minimally impacted endogenous cystine levels. The absolute bioavailability of cystine was 18.6%, 15.1% and 25.6% at doses of 25, 50 and 100 mg/kg, respectively. Intravenously injected D<sub>4</sub>-cystine resulted in dramatically high plasma levels with reduced levels in the brain and liver. Intragastrically administered D<sub>4</sub>-cystine resulted in high levels in the plasma and stomach with relatively low levels in the lung, kidney, heart and brain.

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## 1. Introduction

Glutathione (GSH) is an antioxidant that acts against oxidative stress in vivo. Limited antioxidant capacity is mainly attributed to deficiencies in GSH and/or its precursor cysteine [1]. In addition, GSH plays a central role in affecting the immune response [2]. GSH deficiency can cause liver diseases and cancer. Cystine has various benefits, such as antioxidative, improved liver function and promotion of cellular redox effects. Cystine deficiency can lead to liver necrosis [3], and liver injury can be attenuated via supplementation with cystine in food [4]. Thus, clinically, cystine is used as an auxiliary treatment for hepatitis [5]. T cells need exogenous cystine to generate enough GSH to proliferate [6]. As a precursor, cystine contributes to GSH synthesis [7,8] thereby serving as a treatment for hepatitis and other immune diseases. Cystine exhibits advantages over cysteine as a prodrug given its low oral toxicity and ability to stably decompose to cysteine in cells [9,10], which is the rate-limiting step of GSH synthesis [11–13].

Several oral cystine drugs, such as cystine tablets (Chinese drug

approval number h14023927) and F1 (a GSH precursor with cystine replacing cysteine and selenium added, FT061452/RE39734<sup>TM</sup>), are currently on the market [14]. However, their pharmacokinetic characteristics and tissue distribution are unknown after absorption in the intestine [15]. To date, there is only one report on the absorption and pharmacokinetic properties of oral cystine combined with other amino acids in pigs, but not of cystine alone [16]. Therefore, it is necessary to study and evaluate the pharmacokinetics (PK) and absolute bioavailability of cystine. Cystine is naturally a unique amino acid with rather poor solubility either in water or organic solvents. To facilitate the infusion application of cystine clinically, a stable, low-haemolytic and low-irritant solution of cystine was prepared for this study [17].

Previously, quantitative methods were developed to determine the concentration of cystine. High performance liquid chromatography (HPLC) involves a tedious process with complicated derivatization reagents to increase chromatographic retention and the intensity of ultraviolet spectrum. The unstable derivatization reagents [18] pose a fatal limitation. Mass spectrometry [19–24] was also used for detecting cystine in various samples. Some of these methods could not reach the lower limit of quantification. The need for special instruments also limits the popularization of these analytical methods. By using stable deuterium isotope-labelled

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<sup>\*</sup> Corresponding author.E-mail address: [jiyea@cpu.edu.cn](mailto:jiyea@cpu.edu.cn) (J. Aa).

cystine (D<sub>4</sub>-cystine), in this study, we developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method and evaluated the PK and bioavailability after oral (i.g.) and intravenous (i.v.) administration of an aqueous cystine solution.

## 2. Materials and method

### 2.1. Reagents and materials

3,3,3',3'-D<sub>4</sub>-cystine (98%) and <sup>15</sup>N<sub>2</sub>-cystine (98%) were purchased from Cambridge Isotope Laboratories, Inc. (Cambridge, MA, USA). Cystine and N-ethylmaleimide (NEM) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Methanol (HPLC-grade) was obtained from Merck (Merck, Germany). Formic acid (HPLC-grade) was purchased from Acros (Acros, Belgium). Deionized water was prepared by a Milli-Q ultra-pure water purification system (Millipore, Bedford, MA, USA). Hydrochloric acid (HCl) saturated n-butanol solution was self-formulated. All other chemicals and solvents were of analytical grade.

### 2.2. Animals

Kunming (KM) mice (half male and half female, 20–30 g, 4–5 weeks old) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, Liaoning, China). The use and treatment of mice complied with the Guidance for Ethical Treatment of Laboratory Animals (The Ministry of Science and Technology of China, 2006). Mice were fed standard food and water ad libitum for one week to adapt to the conditions. After fasting overnight, mice were subjected to random grouping and administered the drug as described in Section 2.9. The animal study satisfied the requirements of the Animal Ethics Committee of China Pharmaceutical University (Permit number: 20190625-LSN-Mice200).

### 2.3. Chromatographic conditions

In this experiment, a Shimadzu LC system (Kyoto, Japan) consisted of binary LC-20AD XR pumps, a degasser, a Shimadzu SIL-20AC autosampler and a CTO-20AC column oven. An Agilent ZORBAX 300SB-C<sub>18</sub> column (2.1 mm × 100 mm, 3.5 μm) was used for chromatographic separation at 40 °C eluted with a mobile phase of deionized water supplemented with 0.1% (V/V) formic acid (solvent A) and methanol (solvent B). With a total flow of 0.2 mL/min, the following gradient of elution was performed: 0–1 min, 5% B; 1–2 min, 5%–40% B; 2–7 min, 40% B; 7–8 min, 40%–5% B; and 8–9 min, 5% B. After a batch of 100 samples was injected, the column was washed with 90% methanol for 90 min. It effectively removed the retained compounds and refreshed the column.

### 2.4. Mass spectrometric conditions

The parameters for the mass spectrometer equipped with a heated electrospray ionization source (AB SCIEX QTRAP 5500 system, Sciex, USA) were optimized for cystine, D<sub>4</sub>-cystine and <sup>15</sup>N<sub>2</sub>-cystine (internal standard, IS) to obtain optimal spectral profiles. Analyst 1.6.1 software was installed to acquire and manage the data. In positive ionization mode, multiple-reaction monitoring (MRM) mode was used to monitor the analytes and IS.

The mass spectrometric conditions were as follows: ion spray voltage, 5000 V; ion source gas 1 (N<sub>2</sub>), 55 Arb; ion source gas 2 (N<sub>2</sub>), 50 Arb; source gas temperature, 500 °C; curtain gas (N<sub>2</sub>), 35 Arb; collision gas (N<sub>2</sub>), medium; entrance potential, 10 V; and cell exit potential, 6 V. The selected mass transitions were *m/z* 353.1 → 208.1 for cystine, *m/z* 357.1 → 210.1 for D<sub>4</sub>-cystine, and *m/z* 355.2 → 209.1 for <sup>15</sup>N<sub>2</sub>-cystine. The declustering potential (DP) was set at

63 V for D<sub>4</sub>-cystine and the IS, and 68 V for cystine. The collision energy (CE) for each analyte was 20 eV.

### 2.5. Preparation of stock solutions, calibration standards and control samples

Briefly, 10 mg of cystine, D<sub>4</sub>-cystine and <sup>15</sup>N<sub>2</sub>-cystine were separately dissolved in 1 mL of 0.1 M HCl. The stock solutions showed rather stable at 4 °C for three weeks compared with those freshly prepared (Table S1). 300 mg of NEM was dissolved in 50 mL of deionized water. Subsequently, to obtain working solutions, the stock solution of D<sub>4</sub>-cystine was diluted with 0.1 M HCl to yield a series of concentrations as follows: 10, 20, 50, 100, 200 and 500 ng/mL as well as 1, 2, 5, 10, 20 and 50 μg/mL. In addition, quality control (QC) working solutions at concentrations of 100 ng/mL, 2 μg/mL and 40 μg/mL were prepared separately from a new stock solution for plasma analysis. Then, 100 ng/mL, 1 μg/mL and 15 μg/mL solutions were prepared for stomach, intestine and liver analysis. 20 ng/mL, 400 ng/mL and 8 μg/mL solutions were prepared for lung, kidney, brain and heart analysis. As an IS, <sup>15</sup>N<sub>2</sub>-cystine was diluted with 0.1 M HCl to 3 μg/mL. And NEM was diluted with deionized water to 3 mg/mL. All solutions were stored in a 4 °C refrigerator.

The final calibration standards of 5–5000 ng/mL were obtained by diluting 3 μL of the working solutions with 27 μL of blank mouse plasma. Similarly, the blank tissue homogenate was prepared with the same volume to obtain final concentrations of 5–2000 ng/mL and 1–1000 ng/mL. QC samples were prepared in the same way by diluting with blank plasma and tissue homogenate to obtain low, medium and high concentrations independently.

### 2.6. Sample preparation

#### 2.6.1. Plasma samples

After optimization based on the original method [20], the samples were prepared as follows. Protein precipitation was used during sample preparation. After adding the NEM solution (30 μL) to avoid interference from cysteine, samples (30 μL) were mixed with the IS (10 μL) and methanol (300 μL). The mixture was vortexed for 5 min and then centrifuged twice at a speed of 30,000 g for 5 min at 4 °C (Thermo Sorvall Biofuge Stratos, Germany). A volume of 150 μL of the supernatant was transferred into a new tube and evaporated until dry with nitrogen flow. After the addition of 200 μL of HCl-saturated n-butanol, the mixture was incubated at 65 °C for 40 min. After being subjected to a gentle stream of nitrogen, the dried residue was reconstituted in 150 μL of 0.1% (V/V) formic acid. Following centrifugation, 5 μL was injected for LC-MS/MS analysis.

#### 2.6.2. Tissue samples

Briefly, 0.1 g of each tissue was accurately weighed and homogenized with 1 mL of deionized water containing 3 mg/mL NEM (ULTRA-TURRAX T25 homogenizer, JANKE & KUNKEL IKA®–Labortechnik, Germany) at 13,500 rpm in ice bath four times, each for 30 s at an interval of 10 s. Then, 30 μL of supernatant was used for subsequent preparation, which did not differ from the plasma preparation described above.

### 2.7. Response factor (RF)

A surrogate analyte of D<sub>4</sub>-cystine with properties similar to those of endogenous cystine was employed to generate calibration curves for the quantitative measurement of the endogenous cystine. The RF was used to eliminate isotope effects that could lead to different ionization efficiencies of the isotope and prototype. The RF was calculated by comparing the peak area of isotopic cystine

with that of the corresponding authentic analyte at QC concentrations (final concentrations: 10, 200 and 4000 ng/mL,  $n=4$ ). The values calculated were supposed to be constant over the calibration range. Consequently, the standard curves of surrogate analyte could be used to back calculate the concentration of endogenous cystine; otherwise, the RF value should be incorporated into the calculation [25] (Eq. (1)):

$$a \times \text{Conc.}_{\text{cystine}} = (\text{Area of cystine}/\text{Area of I.S.}) \times \text{RF} - b$$

where  $b$  is the intercept of the calibration curve, and  $a$  is the slope.

## 2.8. Method validation

The method validation assay was strictly executed following the Bioanalytical Method Validation Guidance for Industry [26].

### 2.8.1. Specificity and selectivity

Blank plasma and tissue homogenate from six different mice were processed as described in Section 2.6, and then injected into the instrument to assess the endogenous interference at the retention time of the analyte and IS.

### 2.8.2. Linearity, lower limit of quantification (LLOQ), precision and accuracy

Area ratios of the analyte to IS ( $Y$ ) were used to establish calibration curves with the standard concentration ( $X$ ) by linear regression (weight coefficient  $W=1/X$ ). The linearity range for plasma was 5–5000 ng/mL. And for stomach, intestine and liver, it was 5–2000 ng/mL. The lung, kidney, brain and heart samples had a linearity range of 1–1000 ng/mL. The LLOQ was regarded as the lowest concentration of the calibration curve ( $S/N > 10$ ). Both precision (relative standard deviation (RSD), %) and accuracy (relative error (RE), %) were limited to 20%.

Precision and accuracy were determined using three QC concentrations.  $D_4$ -cystine was added to blank mouse plasma to obtain final concentrations of 10, 200 and 4000 ng/mL and added to tissue homogenate to generate concentrations of 2, 10, 40, 100, 800 and 1500 ng/mL. Six samples were prepared in parallel for each concentration and analyzed in three batches.  $D_4$ -cystine concentrations were calculated based on the standard curve of each batch. Intra- and inter-batch coefficients of variation were obtained.

### 2.8.3. Recovery and matrix effect

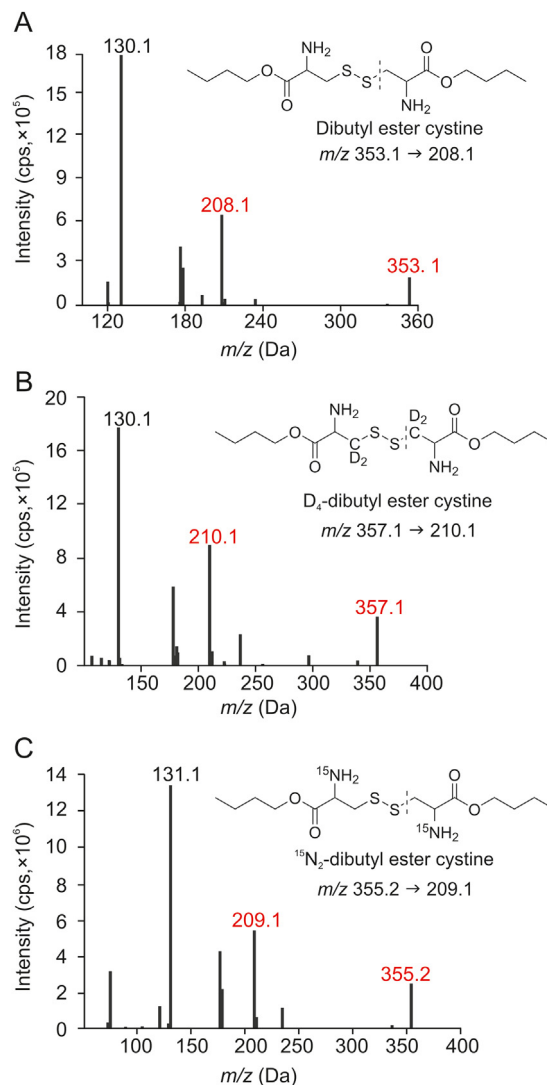
Recovery measures the extraction efficiency of sample preparation. The peak area ratio of analyte to IS was selected as an indication of recovery. Recovery was measured by comparing the peak area ratio of the matrix containing the analyte with that of the extracted blank samples containing the post-spiked analyte. The matrix effect was calculated by comparing the peak area ratio of the extracted blank matrix post-spiked with analyte with that of pure solution spiked with the same nominal concentration of the analyte.

### 2.8.4. Stability

To evaluate the storage and handling of the samples obtained, the investigation of analyte stability in the biological matrix included short-term stability (stored at room temperature for 12 h), post-preparation stability (stored in the auto-sampler at 4 °C for 12 h), freeze-thaw stability (subjected to three freeze-thaw cycles) and long-term stability (frozen at –70 °C for 7 days).

### 2.8.5. Dilution integrity

The biological samples containing 0.5, 10 and 200 µg/mL  $D_4$ -cystine were diluted 50 times with deionized water containing



**Fig. 1.** Mass spectra and chemical structures of derivative products selected for the identification of cystine,  $D_4$ -cystine and the IS ( $^{15}\text{N}_2$ -cystine): (A) dibutyl ester cystine, a disubstituted derivative of cystine; (B)  $D_4$ -dibutyl ester cystine, a disubstituted derivative of  $D_4$ -cystine; and (C)  $^{15}\text{N}_2$ -dibutyl ester cystine, a disubstituted derivative of  $^{15}\text{N}_2$ -cystine.

3 mg/mL NEM. The dilution integrity was assessed based on the analysis and calculation.

## 2.9. Pharmacokinetic study and tissue distribution

Doses (25, 50 and 100 mg/kg) were determined according to clinical application. Meglumine was weighed to prepare a solution in saline (4:100,  $m/m$ ), and then an appropriate amount of  $D_4$ -cystine was dissolved to prepare injections with final concentrations of 2.5, 5 and 10 mg/mL. In addition,  $D_4$ -cystine was suspended in 0.5% carboxymethyl cellulose sodium salt (CMC-Na) to yield a suspension of 5 mg/mL. Approximate 0.25 mL of  $D_4$ -cystine solution was administered via the tail vein or gastrointestinal tract.

A total of 168 mice were used and divided into seven groups (24 mice, 12/sex/group) for the pharmacokinetic study. Three groups received intravenous administration, and three other groups received intragastric administration of the same  $D_4$ -cystine solution, each at 3 doses. The last group of mice was orally given with a suspension solution of  $D_4$ -cystine.

**Table 1**  
Determined response factor (RF) of D<sub>4</sub>-cystine versus cystine at three quality control concentrations (*n*=4).

Conc. (ng/mL)	RF (mean)	RSD (%)
10	1.07	4.10
200	0.97	6.61
4000	1.02	5.18

After i.v. injection of D<sub>4</sub>-cystine (dose: 25, 50 and 100 mg/kg), 100 µL of blood was collected into heparinized tubes from the ophthalmic veins at 3, 5, 10, 20 and 40 min as well as at 1, 1.5, 2 and 3 h post-administration. In each i.v. dose group, 24 mice were divided into four subgroups (*n*=6, three males, three females). The blood samples of the 9 time-points (100 µL per sample) were collected as follows: Sub-group 1, 3 min, 40 min and 3 h; Sub-group 2, 5 min and 1 h; Sub-group 3, 10 min and 1.5 h; and Sub-group 4, 20 min and 2 h. After i.g. administration, blood samples were collected at 5, 10, 15, 20, 30 and 40 min as well as at 1, 1.5, 2 and 3 h post-administration. Three groups were given three doses, the same as the i.v. groups, for a total of 72 mice. The remaining group was orally administered a dose of 50 mg/kg (suspended in 0.5% CMC-Na), with a total of 24 mice. In each i.g. group, 24 mice were divided into four subgroups (*n*=6). The blood samples of the 10 time-points (100 µL per sample) were collected as follows: Sub-group 1, 5 min, 30 min and 2 h; Sub-group 2, 10 min, 40 min and 3 h; Sub-group 3, 15 min and 1 h; and Sub-group 4, 20 min and 1.5 h. 30 µL of plasma obtained was transferred to new tubes pre-filled with 30 µL of 3 mg/mL NEM solution and stored at -70 °C.

For the tissue distribution study, the stomach, intestine, liver, lung, kidney, brain and heart samples were collected at three time points (10 min, 40 min and 2 h) after a high dose (100 mg/kg) was orally administered or intravenously injected. Four mice were included at each time point (two males, two females). After washing with cold 0.9% saline solution, tissues were blotted with filter paper and stored at -70 °C until further analysis. The tissues used for distribution assessment were handled according to descriptions provided in Section 2.6.

### 3. Results and discussion

#### 3.1. Method development

##### 3.1.1. Choice of internal standard

Cystine is an endogenous compound, so it is important to distinguish cystine administered from that present in vivo. In addition, the complex matrix effect in vivo is significantly important in analysis and quantification. In general, stable isotope-labelled analogues are widely utilized due to their similar chemical and physical properties to unlabelled analytes. Sample matrices affect labelled and unlabelled compounds equivalently [27,28]. Therefore, stable isotope-labelled compounds were chosen for administration and as an internal standard. The results indicate that the isotopically labelled compounds exhibit accuracy and stability, meeting the requirements of quantitative analysis.

##### 3.1.2. Sample preparation

Because cystine is minimally soluble in water, we attempted to dissolve it in various reagents. Cystine only exhibited a stable peak in the HCl solution. It is also difficult to dissolve cystine in organic reagents. We tried methanol/acetonitrile precipitation and liquid-liquid extraction. The results showed that only protein precipitation with methanol effectively extracted cystine from biological samples.

##### 3.1.3. Optimization of derivatization conditions

Derivatization of cystine with n-butanol has been reported, although there is no verification or explanation of the products. Under the recommended conditions, investigation and optimization were conducted to explore how the various conditions affect products. Specifically, the composition of the derivatization reagent, the temperature of derivatization and the amount of time were assessed. Briefly, 1 µg/mL D<sub>4</sub>-cystine and 3 µg/mL <sup>15</sup>N<sub>2</sub>-cystine were tested. After the primary screening (Fig. S1), the narrow ranges were identified, and an orthogonal table was designed. The area ratios of several derivative products to <sup>15</sup>N<sub>2</sub>-dibutyl ester cystine (derivative product of the IS) were evaluated. The orthogonal table L9 (3<sup>3</sup>) is presented in Table S2. The results are displayed in Table S3, which illustrates that HCl-saturated n-butanol, 65 °C and 40 min are the optimal reaction conditions to yield relatively few by-products and a suitable response of D<sub>4</sub>-dibutyl ester cystine.

Compared with the negative ionization mode, positive ionization mode produced protonated molecule ions [M+H]<sup>+</sup>, which were chosen as precursor ions, as they had higher intensity. The disubstituted derivatives were selected as optimal ion pairs. We compared two selected reaction monitoring (SRM) transitions of each analyte (Table S4). Secondary fragment ions were selected for better separation, little interference and high response. As there was interference in SRM transition 2 (Fig. S2), SRM transition 1 was selected for the determination of the analytes and was determined as follows: *m/z* 353.1 → 208.1 for cystine, *m/z* 357.1 → 210.1 for D<sub>4</sub>-cystine and *m/z* 355.2 → 209.1 for <sup>15</sup>N<sub>2</sub>-cystine (Fig. 1).

#### 3.2. Method validation

##### 3.2.1. Response factor

The constant response factors of D<sub>4</sub>-cystine versus cystine over the linear range indicated an identical ionization efficiency of these two compounds (Table 1).

##### 3.2.2. Specificity

The extracts, including blank matrices, blank matrices spiked with 3 µg/mL IS, plasma collected 30 min after a low dose of i.g. administration and tissues obtained 40 min after i.v. injection, were analyzed to evaluate the specificity. The chromatograms of D<sub>4</sub>-cystine and the IS obtained under the established chromatographic conditions demonstrated that endogenous substances did not interfere with the target peaks. The retention times of both the D<sub>4</sub>-cystine and IS were the same (5.70 min) in plasma (Fig. 2). The observations reveal that the specificity assessment results met the above-stated requirements.

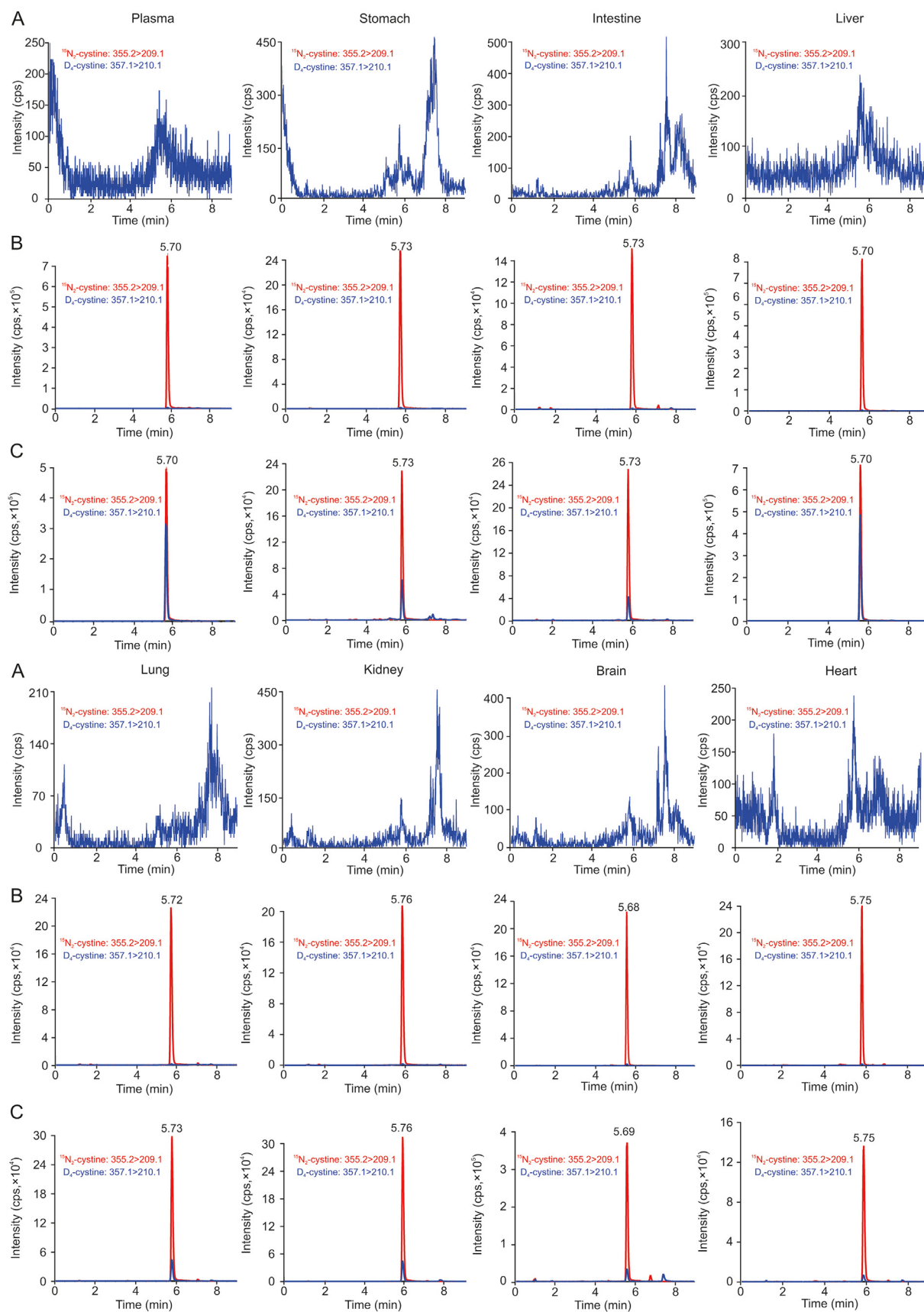
##### 3.2.3. Linearity, LLOQ, precision and accuracy

In the range of 5–5000 ng/mL, the peak area ratio of analyte to IS (Y) in plasma was directly proportional to the concentration (X) using a weight factor of 1/X ( $Y = 0.000974X + 0.00364$ ,  $R = 0.9986$ ). The calibration curves were linear over the range of 5–2000 ng/mL in the stomach, intestine and liver samples and over the range of 1–1000 ng/mL in the lung, kidney, brain and heart samples. All correlation coefficients were acceptable ( $R > 0.99$ ), as shown in Table S5. The LLOQs of the analyte in plasma and tissues were defined by the analytical requirement of  $S/N > 10$  ( $RE \leq 20\%$ ). The measured inter- and intra-batch concentrations met the acceptable limits for both accuracy and precision (Table S6).

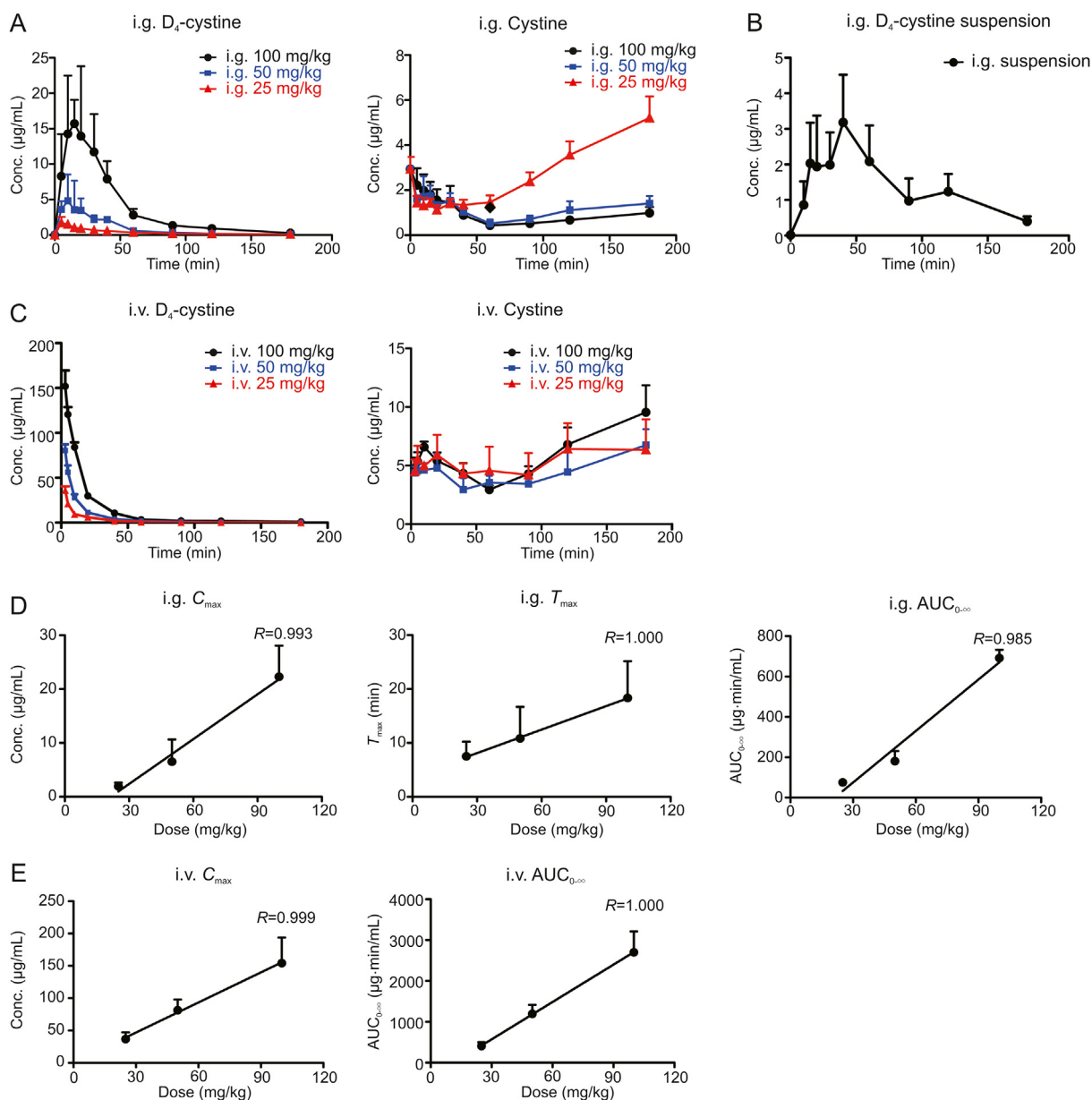
##### 3.2.4. Matrix effect and recovery

The results of the recovery and matrix effect of D<sub>4</sub>-cystine in mice are summarized in Table S7. The average extraction recovery of QC concentrations was over 90%. The statistical analysis demonstrated that the matrix effect could be ignored.





**Fig. 2.** Typical chromatograms for the determination of  $D_4$ -cystine and  $^{15}N_2$ -cystine (IS) in different matrices (plasma and tissues): (A) blank matrix; (B) blank matrix spiked with IS (3  $\mu$ g/mL); and (C) samples (plasma at 30 min after i.g. 25 mg/kg of  $D_4$ -cystine; tissues at 40 min after i.v. 100 mg/kg of  $D_4$ -cystine).



**Fig. 3.** Mean plasma concentration-time profiles of D<sub>4</sub>-cystine and endogenous cystine after administration of D<sub>4</sub>-cystine (mean ± SD, n=6): (A) i.g. doses of 25, 50, and 100 mg/kg; (B) i.g. suspension at 50 mg/kg; (C) i.v. doses of 25, 50, 100 mg/kg; (D) the linear correlation for i.g. administration of C<sub>max</sub>/T<sub>max</sub>/AUC<sub>0-∞</sub> and dose; (E) the linear correlation for i.v. administration of C<sub>max</sub>/AUC<sub>0-∞</sub> and dose.

### 3.2.5. Stability

The stability of D<sub>4</sub>-cystine in plasma under various conditions, including room temperature, 4 °C auto-sampler, three freeze-thaw cycles and long-term storage, was assessed. The results are summarized in Table S8. The analyte was confirmed to be stable under standard conditions.

### 3.2.6. Dilution integrity

The 50-fold dilution of the three high concentrations showed accuracy compared to the nominal concentration. The RE calculated was 4.04%–9.67% within ±15% (Table S9).

## 3.3. Pharmacokinetic study

### 3.3.1. Pharmacokinetics and absolute bioavailability

The method validated above could be successfully used for the

determination of D<sub>4</sub>-cystine and cystine concentrations in biological samples of mice. The average drug concentration-time profiles after i.g. and i.v. administration are shown in Fig. 3. The figure shows that the drug levels after oral administration reached a peak rapidly and decreased to less than 1/10 of C<sub>max</sub> within 3 h. The pharmacokinetic parameters were calculated using non-compartmental analysis in WinNonlin PK software and are displayed in Table 2. According to the data, C<sub>max</sub>, T<sub>max</sub> and AUC<sub>0-∞</sub> of D<sub>4</sub>-cystine were positively correlated with the drug dose and are summarized in Fig. 3. In addition, a summary of endogenous cystine is also provided. The administration of D<sub>4</sub>-cystine slightly interfered with the concentration of endogenous cystine by increasing the total amount of cystine and promoting GSH synthesis. With the continuous depletion of cystine for the synthesis of GSH, more endogenous and exogenous cystine was utilized. When the level of GSH increased, the cystine requirement was reduced,

**Table 2**The pharmacokinetic parameters of D<sub>4</sub>-cystine in mice after oral and intravenous administration (mean ± SD, n=6).

Parameters	D <sub>4</sub> -cystine						i.g. D <sub>4</sub> -cystine suspension (50 mg/kg)
	i.g. (mg/kg)			i.v. (mg/kg)			
	25	50	100	25	50	100	
C <sub>max</sub> (µg/mL)	1.9 ± 0.7	6.5 ± 4.1	22.3 ± 5.8	36.8 ± 10.4	81.3 ± 16.6	154.2 ± 39.6	3.6 ± 0.4
T <sub>max</sub> (min)	7.5 ± 2.7	10.8 ± 5.9	18.3 ± 6.8	–	–	–	40.0 ± 12.7
t <sub>1/2</sub> (min)	51.7 ± 15.3	35.6 ± 8.3	34.7 ± 5.1	35.2 ± 19.4	26.5 ± 8.2	24.4 ± 6.4	55.1 ± 35.9
AUC <sub>0–t</sub> (µg·min/mL)	69.4 ± 6.2	175.5 ± 48.4	676.9 ± 41.3	403.6 ± 91.4	1186.6 ± 221.4	2675.1 ± 497.7	247.0 ± 61.2
AUC <sub>0–∞</sub> (µg·min/mL)	75.9 ± 4.9	180.8 ± 49.8	691.7 ± 40.5	408.9 ± 92.0	1195.5 ± 220.3	2700.2 ± 511.0	274.2 ± 64.2
F (%)	18.6 ± 1.2	15.1 ± 4.2	25.6 ± 1.5	–	–	–	22.9 ± 5.4

and cystine depletion was alleviated. Consequently, there was a slight increase in the total level of cystine after it returned to baseline.

The low calculated bioavailability of cystine (18.6%, 15.1% and 25.6%) indicated challenging absorption when it was administered in a monomeric form, as the bioavailability was much lower than the 59% in pigs reported in the literature [16]. The difference can be explained based on three aspects: (1) Absorption capacity varies among species; (2) orally administered cystine alone is difficult to dissolve and subsequently be absorbed, while multiple amino acids facilitate dissolution; and (3) the non-isotopic determination reported in the literature [16] may be affected by endogenous cystine, resulting in deviation.

### 3.3.2. Tissue distribution

The tissue distribution is shown in Fig. 4. In addition to that found in the plasma, stomach and intestine, a small amount of D<sub>4</sub>-cystine was found in the liver, lung, kidney, brain and heart after i.g. administration. This finding indicates that D<sub>4</sub>-cystine is mainly

absorbed into the blood by intestinal tissue. D<sub>4</sub>-cystine may improve liver function by liver distribution.

## 4. Conclusions

This is the first study to establish calibration curves with isotope-labelled cystine for the determination of both exogenous and endogenous cystine. The optimized method can obtain derivative products with fewer impurities, high response and high sensitivity. Methodological validation confirmed the method's specificity, reliability and reproducibility, and the method can be successfully applied in cystine pharmacokinetic studies and tissue distribution in mice. The PK study showed that the pharmacokinetic behaviour of D<sub>4</sub>-cystine was in accordance with linear features within the tested dose range in KM mice. The tissue distribution study showed that D<sub>4</sub>-cystine had effective penetration, thus spreading widely and rapidly.

## Declaration of competing interest

The authors declare that there are no conflicts of interest.

## Acknowledgments

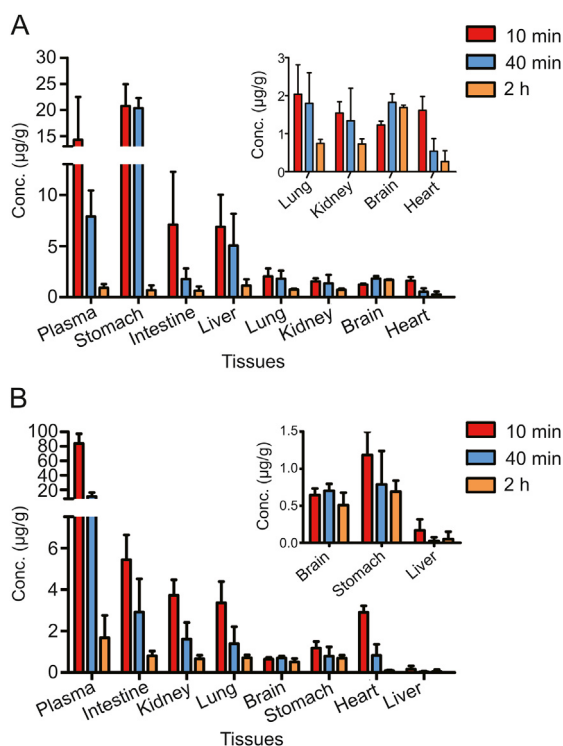
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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jpha.2020.08.010>.

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**Fig. 4.** Tissue distribution of D<sub>4</sub>-cystine after administration of 100 mg/kg D<sub>4</sub>-cystine in mice (mean ± SD, n=4): (A) intragastric administration (i.g.); and (B) intravenous administration (i.v.).

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