Preclinical assessment of the distribution, metabolism, and excretion of S-propargyl-cysteine, a novel H₂S donor, in Sprague-Dawley rats

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Aim: To study the distribution, metabolism and excretion of S-propargyl-cysteine (SPRC), a novel hydrogen sulfide (H₂S) donor, after oral administration in rats.

Methods: Adult Sprague-Dawley rats were used. The tissue distribution of [³⁵S] SPRC-derived radioactivity was measured using a liquid scintillation counter. The plasma protein binding of SPRC was examined using 96-well equilibrium dialysis. The excretion of SPRC in urine, bile and feces was analyzed using the LC-MS/MS method. The major metabolites in rat biomatrices were identified using MRM information-dependent, acquisition-enhanced product ion (MRM-IDA-EPI) scans on API 4000QTrap system.

Results: After oral administration of [³⁵S] SPRC at a dose of 75 mg/kg, [³⁵S] SPRC-derived radioactivity displayed broad biological distribution in various tissues of rats, including its target organs (heart and brain) with the highest in kidney. On the other hand, the binding of SPRC to human, rat and dog plasma protein was low. Only 2.18%±0.61% and 0.77%±0.61% of the total SPRC administered was excreted unchanged in the bile and urine. However, neither intact SPRC nor its metabolites were detected in rat feces. The major metabolic pathway in vivo (rat bile, urine, and plasma) was N-acetylation.

Conclusion: The preliminary results suggest that SPRC possesses acceptable pharmacokinetic properties in rats.

Keywords: hydrogen sulfide (H₂S); S-propargyl-cysteine; pharmacokinetics; distribution; metabolism; excretion; disposition
associated with the prevention of oxidative stress[10]. SPRC also attenuated LPS-induced H9c2 cell activation, which would be beneficial for either the prevention or the treatment of cardiovascular inflammatory disease[11]. Furthermore, the protective effects of SPRC in neuroinflammation in vitro and in vivo indicate therapeutic potential for neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, ischemic stroke, multiple sclerosis, and amyotrophic lateral sclerosis[12-13]. Ma et al reported that SPRC exhibited antitumor activities in SGC7901 gastric cancer cells and confirmed the in vivo antineoplastic effect of SPRC in a nude mice xenograft model[14]. These studies show that SPRC is emerging as a promising H₂S-based therapeutic agent and candidate for future pharmaceutical development.

Preclinical pharmacokinetic characterization of a new drug candidate is an integral part of the drug discovery and development process. Such studies may also provide an in-depth understanding of the drug's mechanism of action. Unfavorable pharmacokinetic properties may lead to drug toxicity, potentially resulting in termination of the program or re-optimization of the chemical structure. Previous studies showed that SPRC was rapidly absorbed and bioavailable in rats[15]. Further studies characterizing the disposition of SPRC in vivo are needed to clarify its pharmacokinetic properties. The aim of this study is to investigate the distribution, metabolism, and excretion of SPRC in rats.

Materials and methods

Chemicals and reagents

SPRC was synthesized by reacting L-cysteine with propargyl bromide. The product was purified by recrystallization from an ethanol-water solution. The final product was verified by 1H nuclear magnetic resonance spectroscopy. The purity was 99.7%, as measured by high-performance liquid chromatography.

[35S] cysteine (>99% radiochemical purity). Ultima-Gold scintillation fluid, and a Soluene-350 tissue solubilizer were all purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). [35S] SPRC (99% purity) was synthesized by reacting [35S] cysteine with propargyl bromide as described above.

Blank Sprague-Dawley rat plasma was obtained from healthy, drug-free rats. Blank dog plasma was purchased from the Shanghai ChemPartner Co LTD (Shanghai, China). Blank human plasma was kindly provided by the Ruijin Hospital (Shanghai, China). Dialysis membranes used for the experiments had a 12 to 14 kDa molecular mass cutoff and were purchased from HTDialysis, LLC (Gales Ferry, CT, USA). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Other reagents used in this study had the highest purity commercially available.

Animal experiments

The experiments were performed in accordance with the Guidelines for Animal Experimentation of Fudan University (Shanghai, China). The protocols used in this study were approved by the Animal Ethics Committee of Fudan University (Shanghai, China). Adult Sprague-Dawley rats (body weight of 200±20 g, half males and half females) were purchased from Sino-British Sipper/BK Lab Animal Ltd (Shanghai, China; animal certificate number: SCXK Sh (2017-0016). The animals were maintained on a 12-h light/dark cycle under controlled conditions (temperature: 20±2°C, relative humidity: 50%-±20%). The rats had free access to food and water throughout the study unless specifically indicated.

In the biliary excretion study, rats were implanted with a cannula that was inserted into the bile duct under anesthesia with diethyl ether, and then allowed to recover before drug administration. Blank bile was collected before administration. Cumulative bile samples were collected during specific time intervals from 0-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-8, 8-12, and 12-24 h after drug administration.

Animals were housed in metabolic cages to collect urine and feces. Urine and feces were collected separately before dosing and from 0-6, 6-12, 12-24, 24-36, 36-48, and 48-72 h. The feces samples were homogenized with water. These samples were stored at -70°C until they were analyzed.

Analytical method for SPRC

The feces homogenate, bile, or urine samples (20 µL) was precipitated with acetonitrile containing 0.44 µg/mL internal standard. After centrifugation at 12000×g for 5 min, 5 µL of the supernatant was used for LC-MS/MS analysis.

SPRC concentrations were quantitated using the LC-MS/MS method. The analytical method has been previously validated and reported for determining the concentration of SPRC in rat plasma[15].

Tissue distribution study of [35S] SPRC-derived radioactivity in rats

The tissue distribution of [35S] SPRC-derived radioactivity was evaluated in Sprague-Dawley rats after an oral administration of 75 mg/kg [35S] SPRC solution (25 µCi/mL, 10 g/L). Animals were euthanized at 0.5 h, 1.5 h, and 6 h after drug administration. Following euthanasia, the rats were exsanguinated, and then plasma and tissues (heart, liver, spleen, lung, kidney, brain, stomach, intestine, and muscle) were collected and homogenized (0.25 g/mL).

Aliquots (400 µL) of the tissue samples were digested in 1 mL of Soluene-350 tissue solubilizer for 3 h at 60°C. Ultima Gold scintillation fluid (10 mL) was then added to each sample. For plasma samples, 100 µL of plasma was directly mixed with 10 mL of scintillation fluid. All the sample preparations were stored in the dark for 24 h before analysis. All of the radioactivity measurements were made using a Tricarb 2910TR liquid scintillation analyzer (Perkin-Elmer, Wellesley, MA, USA). The tissue radioactivity levels were expressed as a percent injected dose per gram of tissue (%ID/g).

Measurement of the plasma protein binding of SPRC using 96-well equilibrium dialysis

Protein binding of SPRC to human, rat, and dog plasma was
measured using a 96-well microequilibrium Teflon dialysis device (HTDialysis, LLC, Gales Ferry, CT). Dialysis membranes were soaked in distilled water for 20 min and then soaked in 30% (v/v) ethanol for 15 min. Just prior to use, the membrane was rinsed three times in deionized water and then rinsed once with isotonic sodium phosphate buffer. After assembling the dialysis plates, 110 µL of blank buffer and spiked plasma samples (2, 10, and 50 µg/mL) were added to the receiver and donor side of the equilibrium dialysis block, respectively. The dialysis block was covered with a plastic lid and placed in a shaker (37°C, 100 r/min) for 6 h. Warfarin was used as a positive control.

SPRC samples from both sides of the chamber were measured using the LC-MS/MS method. Percent binding was calculated using the following equation:

\[
\text{Bound } \% = 100 \times (\frac{[\text{Donor}]-[\text{Receiver}]}{[\text{Donor}]})
\]

Metabolite identification using LightSight software

Preliminary metabolites in rat biomatrices were identified using MRM information-dependent, acquisition-enhanced product ion (MRM-IDA-EPI) scans on the API 4000QTrap system. Pooled rat plasma (0–24 h), bile (0–24 h), urine (0–72 h), and feces (0–72 h) samples were precipitated with acetonitrile. The mobile phase consisted of a methanol/ammonium acetate buffer [10 mmol/L, pH=4.0]: 15/85 (v/v). Other analytical conditions were the same as those applied to the quantification of SPRC, described above.

Mass spectrometer conditions were optimized by infusing 5 µg/mL SPRC solution in distilled water via a syringe pump at a 10 µL/min flow rate. The EPI analyte spectra were collected to identify the major fragments of SPRC ([M+H]+=160.1). The three most abundant fragments were m/z 143.0, 114.0, and 97.0. Therefore, three MRM survey channels at 160.1→143.0, 160.1→114.0, and 160.1→97.0 were used to identify the metabolites of SPRC. Metabolite identification was accomplished using LightSight software, by matching the product ions and neutral losses of the EPI spectra of detected metabolites to the parent compound.

The IDA threshold was set to 500 cps, above which the EPI scan was triggered to collect the fragment ion spectra. The EPI scan rate was 4000 amu/s, and the scan range was 80 to 400 amu. The CEs were set at 30 eV, with a CE spread of 15 eV. Other parameters were set as follows: ion source voltage, +4500 V; temperature, 400°C; curtain gas, 15 psi; ion source gas 1, 20 psi; ion source gas 2, 20 psi.

Other types of mass spectrometric analyses, including enhanced mass spectrometry (EMS), precursor ion (PI), and neutral loss (NL) scans, were performed to ensure that unexpected metabolites were not missed.

Data analysis

The amount of SPRC excreted into bile and urine during each time interval was calculated by multiplying the SPRC concentration by the volume of sample. The cumulative amount of SPRC (X) over a certain time period was calculated by adding all excreted amounts within the period. The same data were also expressed (in the form of % of dose) by dividing X with the total dose administered.

A one-way ANOVA, followed by S-N-K multiple comparisons, was used to evaluate the statistical significance of interspecies differences in plasma protein binding. The differences were considered significant when P<0.05.

Results

Tissue distribution of [35S] SPRC-derived radioactivity

The distribution of [35S] SPRC-derived radioactivity in various organs at three time points was determined after an oral administration of a 75 mg/kg [35S] SPRC solution. SPRC-derived radioactivity was broadly distributed in all of the tissues examined (Table 1). At 0.5 h (the T_max in the plasma kinetics), the concentrations observed were in the following order: kidney>plasma>stomach>liver>lung>intestine>spleen>heart>muscle>brain. When compared with the corresponding plasma concentrations, the kidney tissues showed the highest exposure. The mean concentrations in the kidney tissue were 1.1, 1.5, and 1.8 times that present in the plasma at 0.5 h, 1.5 h, and 6 h, respectively. SPRC-related radioactivity in all the other tissues was the same as or lower than the corresponding plasma concentration.

| Tissue       | 0.5 h | 1.5 h | 6 h    |
|--------------|-------|-------|--------|
| Plasma       | 0.40±0.04 | 0.31±0.08 | 0.18±0.07 |
| Brain        | 0.07±0.01 | 0.08±0.02 | 0.05±0.01 |
| Muscle       | 0.11±0.02 | 0.12±0.03 | 0.05±0.02 |
| Heart        | 0.15±0.06 | 0.10±0.02 | 0.05±0.02 |
| Spleen       | 0.15±0.05 | 0.13±0.04 | 0.07±0.02 |
| Kidney       | 0.44±0.19 | 0.47±0.14 | 0.32±0.12 |
| Stomach      | 0.29±0.22 | 0.12±0.03 | 0.08±0.02 |
| Liver        | 0.23±0.04 | 0.21±0.02 | 0.15±0.07 |
| Lung         | 0.19±0.09 | 0.17±0.07 | 0.12±0.06 |
| Intestine    | 0.17±0.06 | 0.09±0.01 | 0.10±0.06 |

Plasma protein binding

The extent of SPRC bound to human, rat, and dog plasma was studied at three concentration levels using a rapid equilibrium dialysis method. The protein binding to the positive control compound (warfarin) was within the normal range (98.4%±1.1%), indicating the reliability of the equilibrium dialysis study. The fraction bound ranged from -0.8% to -14.5% for human, 3.7% to 10.9% for rat, and -5.2% to 0.6% for dog. The total SPRC recovery after dialysis was >98%. No statistical differences were found at the three concentration levels tested. The statistical analysis revealed no significant difference in the plasma protein binding fractions of SPRC among
the three species.

**Excretion of SPRC in rats**

The excretion of unchanged SPRC after oral administration has been studied in rats using the LC-MS/MS method. No unchanged SPRC was found in rat feces up to 72 h post-dose using a relatively sensitive analytical method (the low limit of quantification was 50 ng/mL). After oral administration in rats, only 2.18%±0.61% and 0.77%±0.61% of the dose was excreted in the bile and urine as the parent drug up to 24 and 72 h, respectively (Figure 2). Less than 3% of the oral SPRC dose was recovered unchanged.

**Identification of the major metabolites of SPRC in urine, bile, feces and plasma**

The representative total ion current (TIC) chromatograms of the drug-treated rat biological samples were compared with the corresponding blank samples. Only one metabolite (M1, RT=10.2 min) of SPRC was found in rat urine, bile, and plasma (Figure 3). However, neither the parent drug nor the N-acetylated metabolite was observed in rat feces (data not shown).

The extracted ion chromatograms (EICs) and representative EPI spectra of M1 were also compared with the parent SPRC (Figure 4). M1 was proposed to be an acetylated metabolite because the precursor ion (m/z 202.1) was 42 m/z larger than the corresponding precursor ion (m/z 160.1) of SPRC. In addition, the fragmentation pattern of M1 is similar to that of SPRC, indicating that the same ions (m/z 143, m/z 114, and m/z 97) were found in the EPI spectra.

To ensure that no unexpected metabolites were missed using the MRM survey scan, different types of survey scan triggering EPI method, were used to detect any other metabolites of SPRC. These methods included EMS-EPI, PI-EPI, and NL-EPI. However, no other metabolites were found using these methods (data not shown).

**Discussion**

SPRC, a novel sulfur-containing amino acid derivative, has been proven to be a potent H2S donor. It was selected for development, in part because of its potent in vitro and in vivo biological activities. However, the lack of comprehensive knowledge about the pharmacokinetic properties of SPRC hampers its further development as a new drug candidate. In the present study, we analyzed the pharmacokinetic properties of SPRC, including the tissue distribution, identification of major metabolites, and route of excretion.

SPRC shares pharmacokinetic properties with other cysteine derivatives, such as SAC[16, 17]. These cysteine derivatives were all bioavailable and were absorbed rapidly and easily in the gastrointestinal tract[15]. After oral administration, [35S] SPRC-derived radioactivity was extensively distributed in various tissues. An accumulation of [35S] SPRC-derived radioactivity exceeding plasma concentrations was mainly observed in the kidney at three different time points. The radioactivity in other tissues was approximate to or lower than the observed plasma concentration. Previous studies showed that SAC was retained at a fairly high concentration in the kidney, and it was speculated that SAC was reabsorbed in the kidney[16]. Therefore, the kidney may play a major role in the elimination of cysteine derivatives in rats and deserves further study.

In the rat excretion study, less than 3% of the oral dose of unchanged SPRC was recovered in the urine and bile. This result indicates that the drug is almost completely metabolized before elimination from the body. The major metabolic pathway of SPRC was N-acetylation, which forms the conjugate compound found in urine, bile, and plasma. The N-acetylated SAC was also the major metabolite of SAC[16]. Neither the parent drug nor the N-acetylated metabolite was found in rat feces. Previous studies demonstrated that S-allyl-L-cysteine sulfoxide and N-allyl-L-cysteine sulfoxide were the expected metabolites of SAC in rat urine[18]. However, these sulfoxide metabolites of SPRC were not observed in our experiment.

In summary, this study examined in detail the pharmacokinetic properties of SPRC in Sprague-Dawley rats. Our study examined the tissue distribution, plasma protein binding, excretion, and metabolites of SPRC. The tissue distribution of [35S] SPRC-derived radioactivity in rats was rapid and extensive. SPRC was distributed mainly in the kidney. The levels of human, rat, and dog plasma SPRC protein binding were low. Only approximately 0.77% and 2.18% of the parent SPRC could be recovered after oral administration in rat urine and bile, respectively. The major metabolite of SPRC in rat urine, bile, and plasma was the N-acetylated metabolite. No parent drug or metabolite was found in rat feces. In addition to

![Figure 2](image-url) **Figure 2.** The cumulative excretion of unchanged SPRC in rat bile (A) and urine (B) after oral administration. SPRC concentrations were determined by the LC-MS/MS method. All values are expressed as the mean±SD. (n=6).
increasing the knowledge of SPRC biological activities, this pharmacokinetic study contributes to the further development of this new drug candidate.

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Author contribution
Yuan-ting ZHENG, Yi-zhun ZHU, and Wei-min CAI designed the experiments, Yuan-ting ZHENG, Jian-hua ZHU, Guo MA, Qing ZHU, Ping YANG, Bo TAN, Jin-lan ZHANG, Hai-xing SHEN, and Jia-lin XU performed the experiments, Yuan-ting ZHENG, and Wei-min CAI wrote the manuscript.

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