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Analysis of Sulforaphane by HPLC-MS/MS *in vitro* and *in vivo*: Chemical Stability, Metabolic Rate and Metabolites

H. ZHANG[1,†], S. SUN[1,†], Q.S. CHEN[1], Y.F. CHAI[2], D.X. SUN[3] and G.Q. ZHANG[1,*]

[1]Department of Pharmacy, Eastern Hepatobiliary Surgery Hospital, Shanghai 200438, P.R. China
[2]Department of Pharmaceutical Analysis, School of Pharmacy, Second Military Medical University, Shanghai 200433, P.R. China
[3]Department of Pharmaceutical Science, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109, USA
†The first two authors contributed equally to this work.

*Corresponding author: Fax: +86 21 8875571; Tel: +86 21 81875571; E-mail: gqzhang@smmu.edu.cn

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Sulforaphane, an anticancer compound occurred naturally in the vegetable of broccoli, was easily degraded in thermal and alkaline conditions. In this case, the stability of sulforaphane was investigated in different temperatures and different pH conditions and the result indicated that sulforaphane was unstable in the conditions of temperature > 50 °C and pH > 10. The microsomal stability of sulforaphane in rat liver microsomes was also analyzed. The results indicated that the microsomal stability of sulforaphane is good ($t_{1/2}$ > 60 min). After oral administration of sulforaphane to rats, four metabolites of sulforaphane were identified in rat plasma and the possible formation mechanism of metabolites *in vivo* was further proposed. This study can provide foundation for the further pharmacological study and structure modification of sulforaphane.

Keywords: Sulforaphane, Stability, Metabolite, HPLC-MS/MS.

INTRODUCTION

Sulforaphane (SF) is a cancer chemopreventive agent present in widely consumed cruciferous vegetables such as broccoli[1-3]. A number of reports continue to support that dietary intake of cruciferous vegetables, especially broccoli, can reduce the risk of different types of cancer[4-7]. The cancer chemopreventive characterization has been primarily attributed to isothiocyanates that occur naturally as the glucosinolate precursors in the plant[4,8,9]. In particular, sulforaphane, one of the isothiocyanate compounds, has received extensive attention for its potent chemopreventive activity[10-12].

Considering the important characterization of this compound, numerous analytical methods about sulforaphane, including high-performance liquid chromatography, gas chromatography, gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry (LC-MS) have been developed[13-17]. However, it was found that sulforaphane was unstable at basic and thermal conditions[15,18,19], so it is very important to clarify its chemical stability for the development of analytical method and further pharmacological study. As a kind of oil, GC-MS may be the best analytical method due to its high sensitivity, but sulforaphane was easily degraded in the injection ports of GC/MS whose temperature is high[20-22]. And therefore it is urgent to develop a sensitive and non-destructive method to analyze the chemical stability and metabolites of sulforaphane.

The purpose of the present study was to investigate the chemical stability in thermal and alkaline conditions, the microsomal stability and the metabolites in rat plasma to provide support for further pharmacological study and structure modification of sulforaphane.

EXPERIMENTAL

Sulforaphane and sulforaphene were purchased from LKT laboratories, Inc. (St. Paul, MN, USA). Its purity was checked by nuclear magnetic resonance (NMR) and liquid chromatography-mass spectrometry (LC-MS) method and it was stored at -20 °C. The β-NADPH (N7505-100MG) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Formic acid and acetic acid was obtained from Fluka (Buchs, Switzerland). Ultrapure water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Other reagents were of analytical grade.

Preparation of standard solutions and buffer solutions at different pH: The sulforaphane authentic standard was accurately weighed and dissolved in DMSO to prepare an

appropriate standard solution and then diluted it to 400 and 5 μM by double-distilled water. The aqueous buffer solutions at pH (1.2, 6.8, 7.4, 8, 10 and 12) were prepared according to U.S. Pharmacopeia Convention. All the solutions were stored at 4 °C before experiments.

Instruments and analytical conditions: HPLC analyses were carried out on an Agilent 1200 series liquid chromatography system (Agilent Technologies, USA), containing of a binary pump, an on-line vacuum degasser, a surveyor autosampling system and a column temperature controller and interfaced to an ABI quadruple ion trap mass spectrometer (AB Sciex, CA, USA) *via* an electrospray ion source. HPLC separation was performed on an Agilent Zorbax Extend-C_{18} column (2.1 mm × 50 mm, 5 μm) and eluting with a linear gradient mobile phase system, which consisted of 0.1 % formic acid (v/v) water (A) and 0.1 % formic acid acetonitrile (B), the gradient elution (v:v) according to the following profile: 0-1 min, 5 % B; 1.01-6 min, 90 % B. The flowing rate was set at 0.4 mL/min. The sample injection volume was 5 μL and the column temperature was kept constant at 25 °C throughout the running time of 6 min. The electrospray ion (ESI) mass spectrometry was conducted to detect sulforaphane and its metabolites at positive ion mode. The conditions of mass spectrometry were as follows: source temperature 400 °C; curtain gas 30 psig; collision gas: medium; ionspray voltage (IS) 5500 V; desolvation gas temperature (TEM) 400 °C; ion source gas 1 (GS1) 60 psi; ion source gas 2 (GS2) 40 psi; collision gas (CAD) high; entrance potential (EP) 4 eV; collision energy (CE) 15 eV. The transition ions at *m/z* 178.1 → 114.1 and *m/z* 176.1 → 112.1 were used to detect sulforaphane and sulforaphene (internal standard). A dwell time of 200 msec was used for the transition. EPI mode was selected to analyze the metabolites.

The linearity was investigated by adding sulforaphane and internal standard (IS) at different concentrations into blank rat liver microsome and PBS sample and five replicate analyses were analyzed for each calibration. The correlation coefficient (r) must be > 0.99. The blank rat liver microsome and PBS solutions were analyzed to confirm the absence of endogenous interference. The lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) were calculated with signal-to-noise ratios of 3 and 10, respectively. Precision and accuracy were investigated by determining the sulforaphane samples at three different concentration levels and each concentration was prepared five replicates. The concentration of each analyte was calculated using a calibration curve. The variation of the precision and accuracy should not exceed 15 %. Three groups of samples (A1, A2, A3) at three concentration levels were prepared for the evaluation of recovery (RE) and matrix effect (ME). Five samples at each concentration level were investigated. A1 was prepared by diluting the working solutions with methanol. A2 was composed of blank liver microsomes or PBS and working solutions spiked before precipitation. A3 was pretreated by spiking the working solutions in blank liver microsomes or PBS after precipitation. The extraction recovery and matrix effect were evaluated by comparing the ratio of peak area of three groups of samples. For measurement of sample stability, three sulforaphane samples of each concentration at low, medium and high levels were prepared to analyze at room temperature for 8 h. The analytical results compared to the expected concentrations less than 15 % were acceptable.

Stability of sulforaphane at different pH: At 25 °C, the sulforaphane solution (400 μM) 10 μL was added in 390 μL buffer solution at different pH (1.2, 6.8, 7.4, 8, 10, 12) to obtain the solution at the concentration of 1 μM, then remove 20 μL of aliquots at 0, 0.5,1, 2, 4, 8, 12, 24 h, transfer 30 μL to 1.5 mL centrifuge tubes and add 90 μL quench solvent (acetonitrile) to make a precipitation of phosphate. The samples were centrifuged at 12,000 rpm for 10 min and the supernatants were taken and stored in 4 °C until analysis.

Stability of sulforaphane at different temperatures: The sulforaphane solution (400 μM) 10 μL was added in 390 μL phosphate buffer solution (pH 7.4) at different temperatures (25, 50, 60, 70, 80, 90 °C), then remove 30 μL of aliquots at 0, 0.5,1, 2, 4, 8, 12, 24 h, transfer to 1.5 mL centrifuge tubes and add 90 μL quench solvent (acetonitrile) to make a precipitation of phosphate. The samples were centrifuged at 12,000 rpm for 10 min and the supernatants were taken and stored in 4 °C until analysis.

Determination of microsomal stability: The incubations were performed on ice in some centrifuge tubes. All the following components except β-NADPH, 10 μL rat liver microsome (20 mg/mL), 4 μL sulforaphane solution (100 μM), 366 μL PBS buffer (0.1 M, pH 7.4), were gently vortex to achieve the incubation mixture. For the sample at t = 0, it is achieved through removing 95 μL the incubation mixture, adding 5 μL PBS buffer and mixing with 300 μL acetonitrile. Prior to reaction initiation, the microsomal suspensions and 20 mM β-NADPH were preincubated for 3 min at 37 °C, respectively. Immediately after fortification of 15 μL of 20 mM β-NADPH into the microsomal suspension, 30 μL reaction samples were taken at 5, 10, 15, 20, 30, 45 and 60 min. The reaction samples were taken and mixed with 90 μL acetonitrile in a 1.5 mL centrifuge tube. Samples were vortex for 30 second and centrifuged at approximately 12,000 rpm for 10 min and 100 μL of the supernatant was transferred into an injection vial for HPLC-ESI/MS/MS analysis. Following sample analysis, the analyte peak area ratio was used to determine sulforaphane concentration. All depletion data were fitted to the monoexponential decay model described in equation: $C(t) = C_0e^{-kt}$.

in vitro half-life ($T_{1/2}$) was obtained using equation:

$$T_{1/2} = 0.693/k$$

Analysis of metabolites of sulforaphane in rat plasma: Male Sprague-Dawley (SD) rats weighing 180-220 g were provided by the Experimental Animal Center of the Second Military Medical University (Shanghai, China). Rats were bred in a breeding room at 25 °C, 60 ± 5 % humidity and a 12 h dark-light cycle. They were given access to tap water and normal chow ad libitum. All the experimental animals were housed under the above conditions for 3-day acclimation and fasted overnight before the experiments. The animal facilities and protocols were approved by the Institutional Animal Care and Use Committee. All procedures were in accordance with the National Institute of Health guidelines regarding the principles of animal care. Rats (n = 3) were administered sulforaphane by oral administration of 25 mg/kg and blood samples (500 μL) were collected at 0.5, 1, 2, 4 h after oral administration. Plasma was separated and mixed immediately

by centrifugation and 300 µL mixed plasma was extracted with 600 µL acetonitrile and vortex for 30 second. The supernatant was collected after centrifugation at 12,000 rpm for 10 min. The 600 µL of supernatant was transferred to a clean centrifuge tube and dried in a speed vacuum at medium temperature. The dried sample was reconstituted in 200 µL acetonitrile, vortex for 30 second and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to injection vials for HPLC-ESI/MS/MS metabolite analysis.

RESULTS AND DISCUSSION

Method development: To achieve the best peak resolution and sensitivity, different mobile phase were investigated. Finally, water containing 0.1 % formic acid and acetonitrile containing 0.1 % formic acid were used for the mobile phase. And different gradient elution programs were taken for better resolution of the analytes and the optimal mobile phase composition were as follows: 0-1 min, 5 % B; 1.01-6 min, 90 % B. The MS/MS conditions were optimized to achieve the maximum response signal of all the analytes as well as the reproducibility of the responses. Different extraction and pretreatment methods were studied to achieve the best extract recovery. Finally acetonitrile were chosen to protein precipitation reagent. Before studying the experimental samples of sulforaphane, the specificity of the analytical method was demonstrated by comparing the total base ion chromatograms of pretreated sulforaphane samples with samples treated in different conditions. The corresponding chromatograms of sulforaphane are shown in Fig. 1, showing none of constituents contained in the matrix to interfere with the detection of sulforaphane and its main metabolites.

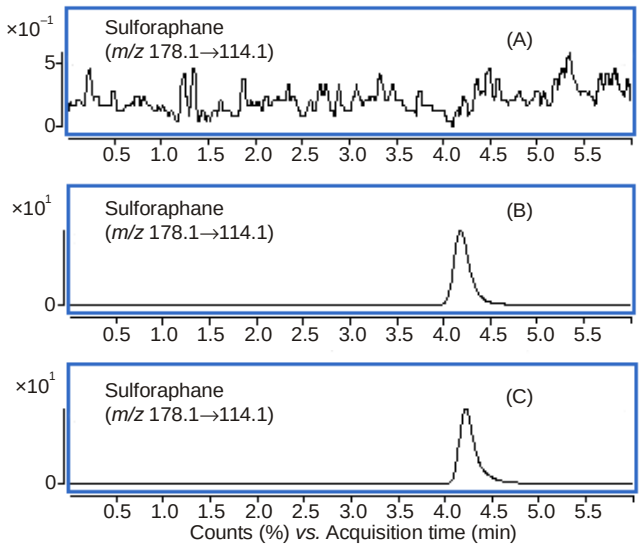

Fig. 1. Representative chromatograms of blank rat liver microsomes (A), spiked standard solution in phosphate buffer solution (B) and rat liver microsome (C)

The methodology was fully validated and the calibration curves were constructed by plotting the peak area *versus* the concentration of each analyte. The correlation coefficient (r) was 0.9978. The lower limits of detection (LLOD) and lower limits of quantification (LLOQ) were 1.074 and 4.096 ng/mL, respectively. The results of the intra-day and inter-day measurements were all less than 10 % (RSD). For stability test, the same sample solution was analyzed every 2 h over 12 h at room temperature. The RSD of contents of sulforaphane in the same sample ranged between 1.28 and 8.65 %, which indicated that the sample was stable over 12 h under the experimental conditions. The samples at three concentrations (n = 9) were then extracted according to the procedure described above and analyzed. The recovery of each component was calculated as the percentage of the net amount of each compound obtained after extraction from that had been added prior to the extraction. The recovery results were all between 89.4 % ± 5.3 and 93.2 % ± 6.2 and within satisfactory ranges. The matrix effect ranged from 89.7 ± 6.6 to 112.4 % ± 4.8 for sulforaphane and the mean matrix effect for IS was 114 % ± 7.2.

Stability of sulforaphane at different pH: At 25 °C and different pH, it was found that sulforaphane was relatively stable when pH was below 10. While pH was above 10, sulforaphane was degraded easily and it was degraded totally within 5 h at pH 12. And therefore sulforaphane is unstable in alkaline conditions. The results are shown in Fig. 2.

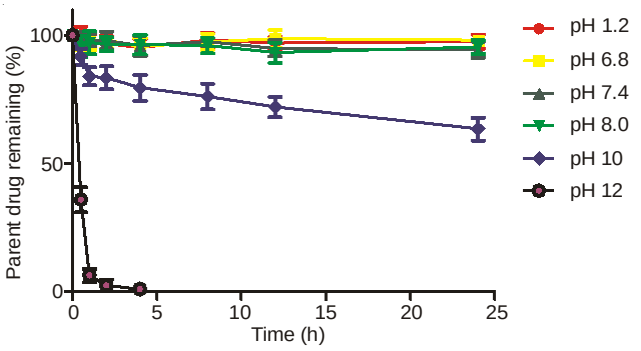

Fig. 2. Degradation profile of sulforaphane in aqueous solution (25 °C) at different pH. Data present in a linear scale and each point represents the mean ± SD of six replicate determinations

Stability of sulforaphane at different temperatures: In pH 7.4 and different temperature conditions, it was found that sulforaphane was stable at 25 °C within 24 h, while sulforaphane became unstable when the temperature was above 50 °C and 50 % of the sulforaphane would be degraded at 90 °C within 4 h. So 90 °C and pH 12 were chosen to analyze the degradation products. The results are shown in Fig. 3.

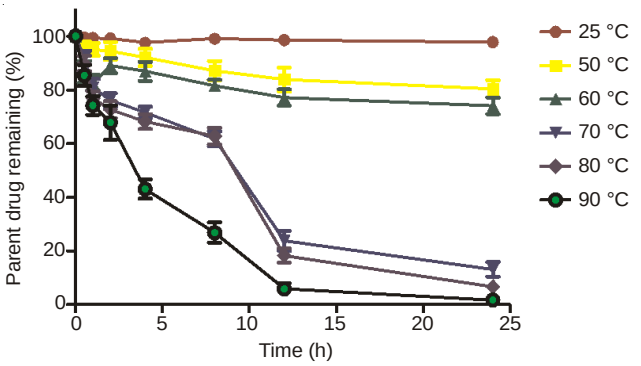

Fig. 3. Degradation profile of sulforaphane in aqueous solution (pH 7.4) at different temperature. Data present in a linear scale and each point represents the mean ± SD of six replicate determinations

Determination of microsomal stability: It is known that metabolism contributes greatly to drug clearance and it influences the systematic drug exposure directly. In this research, the metabolic stability of sulforaphane at 1 μM was evaluated in rat liver microsomes. The data are present in Fig. 4. The *in vitro* $t_{1/2}$ of sulforaphane was more than 60 min. The result suggested that phase-I liver metabolism contributed little to the elimination pathway and was not the main metabolism pathway.

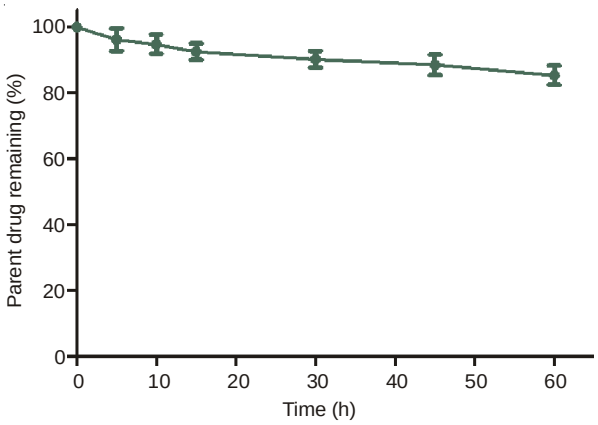

Fig. 4. Elimination profile of sulforaphane in rat liver microsomes. Data present in a linear scale and each point represents the mean ± SD of six replicate determinations

Analysis of sulforaphane metabolites in rat plasma: Blood was collected after oral administration of sulforaphane at a dose of 25 mg/kg and acetonitrile was chosen for protein precipitation reagent. The plasma samples of different time points were mixed together and analyzed by LC-MS/MS. These metabolites were identified based on their retention time on the extend C_{18} column and precursor ion scanning mode. Because the ion at *m/z* 178 of sulforaphane could produce the fragmentation ions at *m/z* 114, 119 and 136, analysis in parent ion scanning mode, with the fragmentation ions at *m/z* 114, 119, 136 and 178 as precursor ions, were performed for the analysis of blank and sample plasma. It was found that the ions at *m/z* 485, 356, 341 and 299 have higher response, which might be the sulforaphane metabolites. The structures of these metabolites were sulforaphane glutathione conjugate (MW 484), sulforaphane cysteinylglycine conjugate (MW 355), sulforaphane N-acetyl-cysteine conjugate (MW 340, sulforaphane cysteine conjugate (MW 298). The possible formation mechanism is listed in Fig. 5.

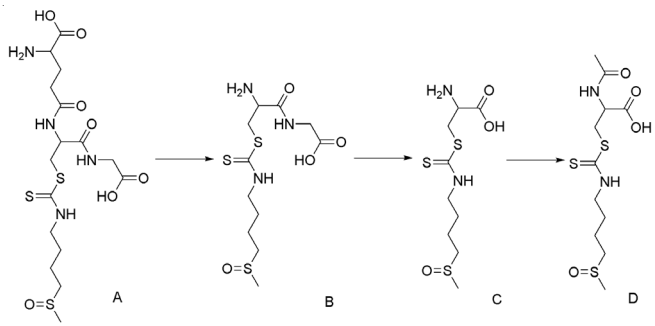

Fig. 5. Biotransformation pathway of sulforaphane in Sprague-Dawley rat; A: sulforaphane gluthatione conjugate; B: sulforaphane cysteinylglycine conjugate; C: sulforaphane cysteine conjugate; D: sulforaphane N-acetyl-cysteine conjugate

Conclusion

A simple and fast LC-MS/MS analytical method with high sensitivity was developed. Using this method, sulforaphane, the stability of sulforaphane at different temperature and pH was investigated and the microsome stability was also explored in rat liver microsomes. Four phase II metabolites were indentified in rat plasma. This research can provide references for the further pharmacological study and structure modification of sulforaphane.

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