Development and Validation of an LC–MS–MS Method for the Simultaneous Determination of Sulforaphane and its Metabolites in Rat Plasma and its Application in Pharmacokinetic Studies

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Abstract

A highly sensitive and simple high-performance liquid chromatographic–tandem mass spectrometric (LC–MS–MS) assay is developed and validated for the quantification of sulforaphane and its metabolites in rat plasma. Sulforaphane (SFN) and its metabolites, sulforaphane glutathione (SFN-GSH) and sulforaphane N-acetyl cysteine (SFN-NAC) conjugates, are extracted from rat plasma by methanol–formic acid (100:0.1, v/v) and analyzed using a reversed-phase gradient elution on a Develosil 3 µm RP-Aqueous C_{30} 140Å column. A 15-min linear gradient with acetonitrile–water (5:95, v/v), containing 10 mM ammonium acetate and 0.2% formic acid, as mobile phase A, and acetonitrile–water (95:5, v/v), containing 10 mM ammonium acetate and 0.2% formic acid as mobile phase B, is used. Sulforaphane and its metabolites are well separated. Sulforaphene is used as the internal standard. The lower limits of quantification are 1 ng/mL for SFN and 10 ng/mL for both SFN-NAC and SFN-GSH. The calibration curves are linear over the concentration range of 25–20,000 ng/mL of plasma for each analyte. This novel LC–MS–MS method shows satisfactory accuracy and precision and is sufficiently sensitive for the performance of pharmacokinetic studies in rats.

Introduction

Epidemiological studies have suggested that consumption of cruciferous vegetables can protect against cancer in humans (1). Cruciferous vegetables are rich in glucosinolates, which are metabolized in the body to isothiocyanates by the enzymatic action of plant-specific myrosinase or intestinal microflora (2). Sulforaphane (SFN) (4-methylsulfinylbutyl isothiocyanate, Figure 1) is a naturally occurring isothiocyanate, which was first identified in broccoli extracts as the principal inducer of the quinone reductase activity (3). Subsequently, numerous cell-based assays and animal studies have demonstrated the strong chemopreventive effects of SFN (3–7). A Phase I clinical trial by Talalay and colleagues had been conducted to evaluate the short-term safety and toxicity of broccoli sprout extracts (7 days of treatment , three doses per day) containing either glucosinolates (principally glucoraphanin, the precursor of SFN) or isothiocyanates (principally SFN) (8). The results showed no significant or consistent abnormal events (toxicities) associated with any of the sprout extract ingestions. However, no plasma pharmacokinetic of SFN and its metabolites sulforaphane glutathione (SFN-GSH) and sulforaphane N-acetyl cysteine (SFN-NAC) have been reported. Such information is necessary to evaluate the concentration/efficacy relationship.

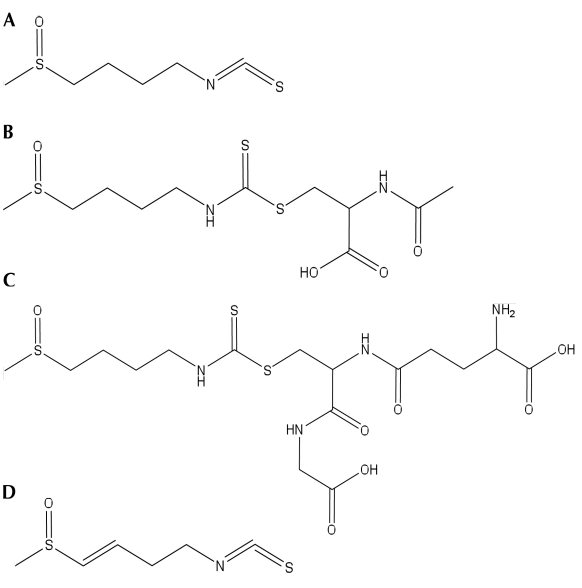

Figure 1. Structures of (A) sulforaphane (SFN), (B) sulforaphane-N-acetyl cysteine (SFN-NAC), (C) sulforaphane-GSH (SFN-GSH), and (D) the internal standard sulforaphene.

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Sulforaphane is metabolized through the mercapturic acid pathway, initially via GSH conjugation, a reaction likely catalyzed by glutathione-S-transferases (GST), which is subsequently metabolized to SFN-cysteine conjugate and finally to SFN-NAC (9). Figure 1 shows the chemical structures of SFN and its major metabolites (1). Thus, the simultaneous determination of the concentrations of SFN, SFN-NAC, and SFN-GSH is crucial in conducting in vivo pharmacokinetic studies.

Very limited analytical methods have been reported for the analysis of SFN and its metabolites in plasma of treated animals for the performance of pharmacokinetic studies. Agrawal et al. (10) used solid-phase extraction to extract SFN metabolites and liquid–liquid extraction using ethyl acetate to extract SFN from rat plasma. The HPLC runtime was 35 min, and a Thermo-Finnigan LCQ Classic detector was used for quantification. This analytical method was used for the analysis of intestinal perfusate and plasma samples from a single-pass intestinal perfusion study with mesenteric vein cannulation in rats. Campas-Baypoli et al. (11) developed and validated an HPLC–UV photodiode array method to determine the SFN level in broccoli by-products. This method is not suitable for the analysis of SFN and its major metabolites in plasma due to the specificity and higher sensitivity required. Al Janobi et al. (12) developed and validated an LC–MS–MS method for the measurement of sulforaphane, iberin, and their mercapturic acid pathway metabolites in human plasma and urine using N-acetyl-S-(N-butylthiocarbomyl)-L-cysteine as the internal standard. In that method, 500 µL of human plasma was used for the analyte quantitation in 13 MRM channels. For the small volume of rat plasma samples as required in this current study, and to assess the matrix effects from rat plasma specifically for SFN, SFN-GSH, and SFN-NAC, a new sensitive and specific bioanalytical method would be needed for our pharmacokinetics study. The current study was initiated to develop and validate a highly sensitive LC–MS–MS method to quantify SFN and its metabolites in rat plasma using protein precipitation. The current method was successfully used to evaluate the pharmacokinetics of SFN and its metabolites in the rats following the intravenous administration of SFN.

Experimental Methods

HPLC–MS–MS analysis

Chemicals and reagents

S,R-Sulforaphane (99% pure) was purchased from LKT (Minneapolis, MN). It was stored at –20°C. SFN-NAC and SFN-GSH were generous gifts from Professor H.Q Tang (Rutgers University). Sprague-Dawley (SD) rat plasma was obtained from Hilltop Lab Animals (Scottdale, PA). HPLC grade acetonitrile, methanol, and ammonium acetate were purchased from Fisher Scientific (Hampton, New Hampshire). Formic acid (99% pure) was purchased from Sigma-Aldrich (St. Louis, MO).

LC–MS–MS instruments and conditions

An Agilent 1100 HPLC system consisting of a binary pump and an autosampler was used (Agilent, Palo Alto, CA). The reverse-phase chromatography was performed with an analytical Develosil C_{30} column (150 × 2.0 mm, 3 µm, Thermo Fisher Scientific, Waltham, MA), which was kept at 30°C, while the autosampler was maintained at 10°C. The optimized method used a binary gradient mobile phase with acetonitrile–water (5:95, v/v) containing 10 mM ammonium acetate and 0.2% formic acid as mobile phase A, and acetonitrile–water (95:5, v/v) containing 10 mM ammonium acetate and 0.2% formic acid as mobile phase B. The gradient program is shown in Table I. The flow rate was 0.25 mL/min and the injection volume was 10 µL.

A MicroMass Quattro Ultima tandem mass spectrophotometer equipped with MassLynx Version 3.5 software was used for the detection and quantification of the analytes (Thermo Fisher Scientific). The MS–MS detection was achieved using a positive ion multiple reaction monitoring (MRM) mode with an *m/z* transitions of 176.1 → 111.5 for sulforaphene, 178 → 113.6 for SFN, 485 → 178 for SNF-GSH, and 340.6 → 178 for SFN-NAC (13). The instrument settings are listed in Table I.

Stock solutions and standards

Primary stock solutions of SFN, SFN-GSH, and SFN-NAC were prepared in methanol, and the stock solutions were stored at –80°C. The primary stock solutions of these analytes were first diluted quantitatively with methanol to give working solutions with concentrations of 25, 50, 100, 250, 500, 1000, 5000, 10000, and 20000 ng/mL for the calibration standard and quality control (QC) samples. The calibration standards were prepared fresh daily by spiking 50 µL blank rat plasma with 5 µL of methanol or analyte working solutions, and 5 µL of sulforaphene solution (IS, 1000 ng/mL). Quantification was achieved by using a weighting factor of $1/\chi 2$.

Table I. HPLC Mobile Phase Gradient Program and MS–MS Conditions for the Analysis of SFN and its Metabolites

HPLC conditions			
Flow rate		0.25 mL/min	
Gradient Program	**Min**	**A**	**B**
	0	92%	8%
	8	0	100%
	8.1	92%	8%
	15	92%	8%
Auto-sampler			
Injection volume		10 µL	
Sample temperature		10°C	
Column temperature		30°C	
MS–MS conditions			
Source		ES+	
Capillary		3.20 kV	
Cone		35 V	
Source Temperature		120°C	
Desolvation Temperature		250°C	
Ion Energy		1.0	
Entrance		–5	
Collision		10	

Sample preparation procedures

Blank rat plasma (50 µL), spiked plasma, or pharmacokinetic study plasma samples were treated twice, each with 200 µL of methanol containing 0.1% formic acid and mixing for 4 min on a cyclomix at room temperature. After centrifugation at 10,000 g for 3 min at 4°C, the supernatant was transferred to a clean tube. The combined supernatant was evaporated to dryness under a stream of nitrogen gas at room temperature. The residue was reconstituted in 100 µL of acetonitrile–water (50:50, v/v), vortexed for 2 min, filtered through a 0.45-µm Nylon spin-filter (Analytical Sales and Services, Pompton Plains, NJ), and transferred into an HPLC sample vial for LC–MS–MS analysis.

LC–MS–MS method validation

Specificity and selectivity

The chromatographic interference from endogenous compounds was assessed by comparing chromatograms of blank rat plasma, plasma spiked with SFN, SFN-NAC, SFN-GSH, or sulforaphene, and plasma samples obtained from SFN pharmacokinetic studies in the rat.

Sensitivity

The lower limit of quantification (LLOQ) was determined during the evaluation of the linear range of the calibration standards. LLOQ was defined as the lowest concentration yielding a precision (%CV) of less than 20% and an accuracy within 20% of the theoretical value (i.e., accuracy between 80% and 120%) for both intra- and inter-day analysis.

Linearity of calibration curve

Calibration curves were obtained by plotting the peak area ratios of each analyte to the internal standard against the theoretical concentrations of the spiked analytes in plasma. The linearity of the calibration curves were evaluated using $1/\chi 2$ as a weighing factor. The minimally acceptable correlation (r^2) for the calibration curves was 0.98.

Precision and accuracy

In order to assess the intra- and inter-day precision and accuracy, SFN, SFN-GSH, and SFN-NAC QC samples at low (50 ng/mL), middle (500 ng/mL), and high (5000 ng/mL) concentrations were prepared as described above. The intra-day precision was assessed by calculating the % CV for the analysis of the QC samples in triplicates; and inter-day precision was determined by the analysis of the QC samples on three separate days. Accuracy was calculated by comparing the averaged measurements to the nominal values, and was expressed in percentage. The criteria for acceptability of the precision were that the % CV for each concentration level should not exceed 15% with the exception of the LLOQ, for which it should not exceed 20%. Similarly, for accuracy the averaged value should be within ± 15% of the nominal concentration with the exception for the LLOQ, where the limit was ± 20%.

Recovery

The recovery for SFN and its metabolites were determined by comparing the peak area ratios of the analytes in rat plasma at the QC concentrations to those in methanol at equivalent concentrations and expressed in percentage.

Stability

The short-term stability of SFN and its metabolites in rat plasma was evaluated by subjecting the QC samples to storage in the HPLC auto-sampler at 10°C followed by injections at 4 and 8 h after the samples were prepared. The stability of the QC samples from plasma was also assessed after three freeze-and-thaw cycles (–80°C). Freezer stability of the analytes in rat plasma was assessed by analyzing the QC samples stored at – 20°C for 3 and 15 days. The peak areas ratios of the analytes at the QC concentration levels at the initial condition were used as reference to determine the relative stability of the analytes.

Pharmacokinetics of SFN in the rats

Male Sprague-Dawley rats weighing between 250 and 300 g with jugular vein cannulae were purchased from Hilltop Lab Animals Inc (Scottdale, PA). The animals were housed in the AAALAC accredited Animal Care Facility of Rutgers University under 12 h light-dark cycles with free access to food and water. Upon arrival, the rats were given AIN-76A diet (Research Diets, NJ, USA) free of antioxidant and acclimatized to the laboratory conditions for 3 days. Rats (n = 4) were given SFN as an intravenous (i.v.) bolus injection at 25 mg/kg in 0.9% saline solution through the jugular vein cannulae, followed by saline solution flushing. Blood samples (200 µL) were collected at 2, 5, 15, 30, and 45 min, and 1, 1.5, 2, 4, 6, 8, 12, and 24 h after SFN administration. Plasma was separated immediately by centrifugation and stored at –80°C, pending analysis.

The SFN and metabolites plasma concentration versus time data were analyzed using WinNonlin 5.2 software (Pharsight, CA) to determine the pharmacokinetic parameters.

Results and Discussion

Method development

The LC conditions used were selected based on the optimization of peak separation, and the MS–MS conditions were set up based on the maximum signal of the analytes as well as the reproducibility of the responses. Specifically, the MRM mode was selected as it provided higher sensitivity and selectivity signals for each of the analytes. The mobile phase contained MS–MS compatible components (i.e., ammonium acetate and formic acid). The flow rate and gradient conditions of the mobile phase were chosen to achieve balanced results in terms of speed, peak shape, resolution, and sensitivity for SFN and its metabolites. Carry over was evaluated by the injection of blank plasma sample extract after the injection of samples at 20,000 ng/mL; no significant carry over (less than 0.1%) was observed.

Protein precipitation was used for sample preparation. Methanol containing formic acid was chosen based on a previous study in which the same solvent was used to extract SFN, SFN-NAC, and SFN-GSH. These conditions of sample preparation and LC–MS–MS analysis enabled the detection of concentrations of SFN as low as 1 ng/mL in 50 µL of rat plasma.

LC–MS–MS method validation

Specificity and selectivity

Figures 2 and 3 represent typical chromatograms of the blank rat plasma and the analytes in the rat plasma sample. Figure 4 shows typical mass spectra at the selected retention times of the analytes, SFN, sulforaphene, SFN-GSH, and SFN-NAC. No interference of endogenous peaks was observed. Typical retention times were: SFN and sulforaphene, 7.6 min, SFN-NAC, 3.5 min., and SFN-GSH, 2.2 min. There were no interfering peaks from blank rat plasma in at least six tests with different sources of plasma.

Sensitivity

The lower limit of quantification was defined as those concentrations and showed 10 times signal-to-noise ratio. The LLOQ in rat plasma were 1 ng/mL for SFN, 10 ng/mL for SFN-GSH, and 10 ng/mL for SFN-NAC.

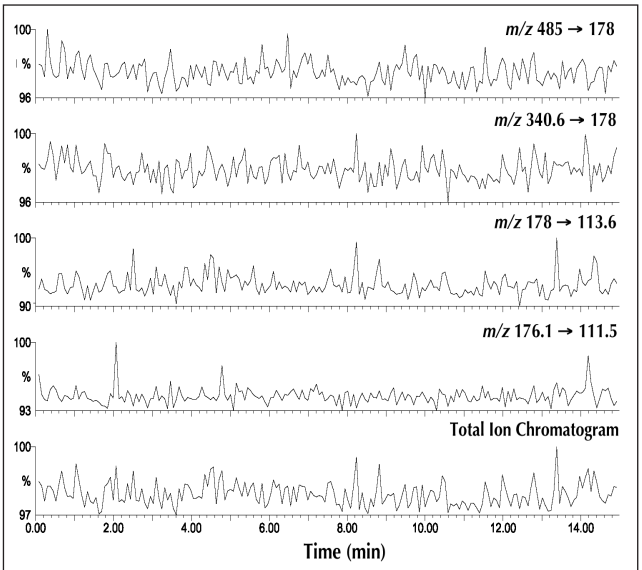

Figure 2. Typical chromatograms of blank plasma after sample processing, showing no interfering peak.

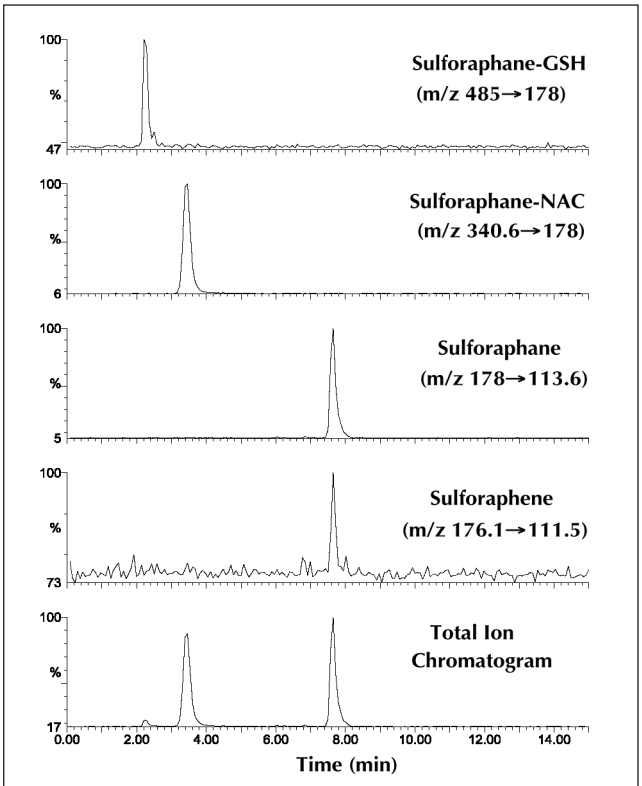

Figure 3. Representative total ion chromatogram (TIC) of the processed plasma sample with four MRM channels for SFN-GSH, SFN-NAC, SFN, and sulforaphene.

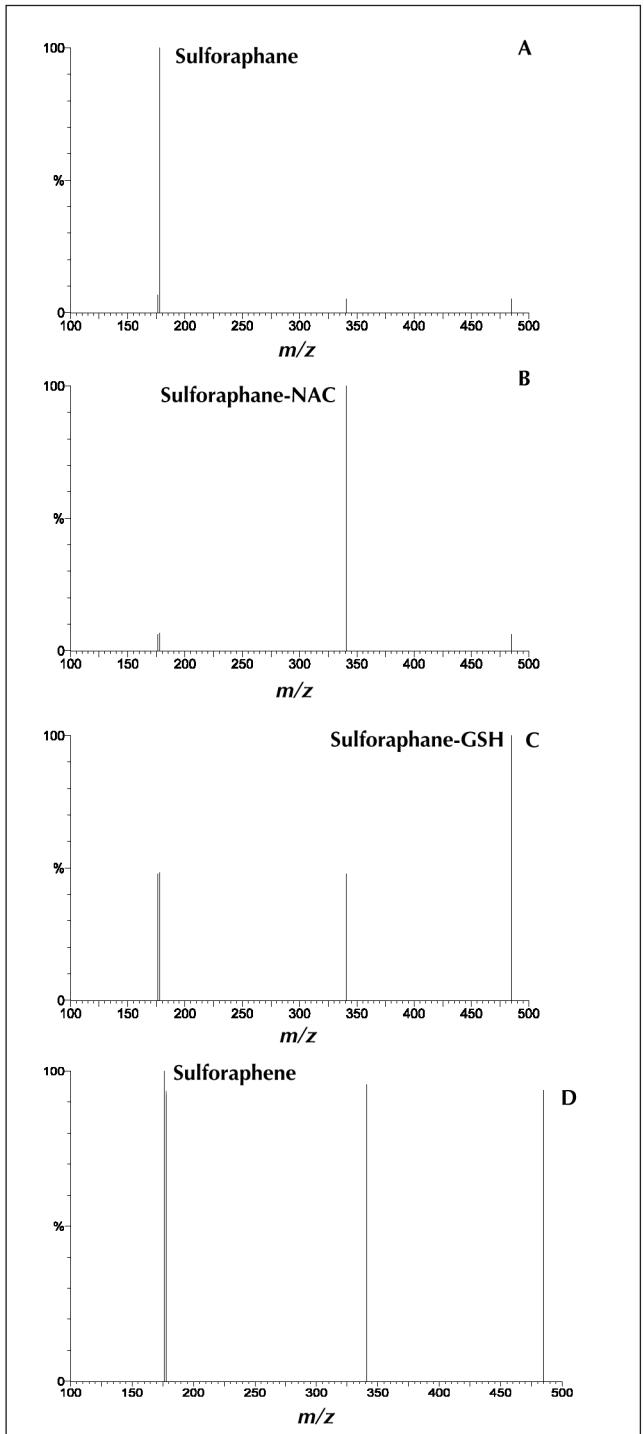

Figure 4. Mass spectra of the analytes, (A) SFN, (B) SFN-NAC, (C) SFN-GSH, and (D) the internal standard of sulforaphene.

Table II. Sulforaphane and its Major Metabolites Quality Control Sample Concentration Levels and the Linearity and Ranges of the Analytical Method

Validation Parameter	SFN-GSH			SFN-NAC			SFN		
QC Levels	Low	Middle	High	Low	Middle	High	Low	Middle	High
QC Conc. (ng/mL)	50	500	5000	50	500	5000	50	500	5000
Linearity Range	25–20,000 ng/mL			25–20,000 ng/mL			25–20,000 ng/mL		
Correlations (r^2)*	0.996 ± 0.006			0.990 ± 0.013			0.995 ± 0.003		

* Of calibration curves ± SD

Table III. Intra-Day and Inter-Day Precision and Accuracy for SFN and Its Metabolites (*n* = 3)

	Nominal Conc. (ng/mL)	Conc. found (ng/mL)	Precision (% CV)	Accuracy (% bias)
Intra-day				
	50	48	9.7	–3.3
Sulforaphane	500	503	1.5	0.6
	5000	4991	0.8	–0.2
	50	46	7.9	–7.8
Sulforaphane-NAC	500	472	6.0	–5.6
	5000	4465	5.9	–10.7
	50	52	3.5	3.8
Sulforaphane-GSH	500	497	9.8	–0.6
	5000	4927	3.1	–1.5
Inter-day				
	50	49	1.7	–2.0
Sulforaphane	500	501	0.2	–0.1
	5000	5017	0.4	0.3
	50	49	3.7	–2.8
Sulforaphane-NAC	500	509	3.2	1.8
	5000	4900	8.4	–2.0
	50	49	9.4	–1.4
Sulforaphane-GSH	500	455	12.3	–9.1
	5000	4413	13.2	–11.7

Table IV. Stability After Three Freeze-Thaw Cycles and After Storage at –20°C for Three or Fifteen Days for the Quality Control (QC) Samples (*n* = 3)

Level (ng/mL)	Stability Condition	Concentration remaining [ng/mL (% Remaining) ± CV%]		
		SFN-GSH†	SFN-NAC	SFN
50	3 freeze-thaw cycles	49.7 (99) ± 11	51.6 (103) ± 3	49.4 (99) ± 3
	3-day (–20°C)	44.2 (88) ± 16	49.8 (100) ± 0.4	51.9 (104) ± 3
	15-day (–20°C)	NT*	53.0 (106.0) ± 21	49.3 (99) ± 4
500	3 freeze-thaw cycles	492.3 (99) ± 10	491.5 (98) ± 3	515.6 (103) ± 8
	3-day (–20°C)	441.4 (88) ± 14	497.3 (100) ± 2	502.8 (102) ± 2
	15-day (–20°C)	NT	487.4 (98) ± 17	521.0 (104) ± 6
5000	3 freeze-thaw cycles	4975.9 (99) ± 4	5060.1 (101) ± 2	5071.2 (101) ± 4
	3-day (–20°C)	4445.6 (89) ± 7	5025.4 (101) ± 1	5030.0 (101) ± 1
	15-day (–20°C)	NT	4596.8 (92) ± 7	5334.3 (107) ± 12

* NT = Not tested.
† SFN-GSH is unstable under the storage conditions and should be analyzed within three days after sample preparation.

Linearity of calibration curve

The calibration curves were linear over the concentration range of 25–20000 ng/mL for SFN, SFN-NAC, and SFN-GSH in rat plasma. The correlation (r^2) of the calibration curves, using $1/\chi 2$ as a weighing factor, and ranges of concentrations used for SFN and its metabolites are shown in Table II.

Precision and accuracy

The precision and accuracy for the analysis of SFN and its metabolites are reported in Table III. The results demonstrated satisfactory intra-day and inter-day precision and accuracy as shown by the CV and the bias values of <15% for the three QC concentration levels.

Recovery

Recovery was evaluated by comparing the analyte peak area ratios of the extracted samples at the three QC levels with standard solutions of equivalent concentrations in methanol. The individual recovery values were 75.2–81.9% for SFN-GSH, 77.5–88.9% for SFN-NAC, and 83.3–86.1% for SFN at the low, middle, and high concentration levels.

Stability

Short-term stability for the extracted plasma samples stored in the HPLC auto-sampler at 10°C was satisfactory. After 4 and 8 h in the auto-sampler, the percents remaining were 102.0% and 101.5% for SFN-GSH; 101.9% and 101.1% for SFN-NAC, and 98.2% and 97.0% for SFN, respectively, compared to samples injected immediately. The stability of SFN and its metabolites under other conditions was evaluated, and the results are listed in Table IV. It was observed that SFN-GSH was unstable and degrades rapidly under these conditions. Therefore, analysis would need to be performed after the samples are prepared without extended storage even at –20°C. SFN and SFN-NAC are relatively more stable after storage at –20°C for 3 days and for 15 days.

Application of the LC–MS–MS method to the pharmacokinetics study

Plasma concentrations of SFN and its metabolites from a pharmacokinetic study in Sprague-Dawley rats were successfully quantified using the developed analytical method. Plasma concentration versus time profiles of the three analytes after intravenous administration of SFN at a 25 mg/kg dose are shown in Figure 5; the basic pharmacokinetic parameters are listed in Table V. The SFN disappearance from plasma showed a faster initial phase which lasted for approximately 4 h, followed by a slower phase with an apparent half-life of approximately 3 h. The compound demonstrated a moderate clearance with a high Vdss. It was also obvious that SFN is quickly metabolized to SFN-GSH and SFN-NAC as indicated by the quick appearance of both metabolites very early after the administration of SFN. The AUC of SFN-GSH and SFN-NAC constituted approximately

12.5% and 9.1% based on the molar ratios of that of SFN, respectively, indicating that these important metabolites are circulating in the rat plasma. Both metabolites were also readily eliminated from plasma with a likely apparent slower half-lives than the parent compound.

Table V. Summary of Pharamcokinetic Parameters of SFN and Metabolites in Sprague-Dawley Rats (*n* = 4)

Parameters	SFN	SFN-NAC	SFN-GSH
$AUC_{0-24\,h}$ (μg × h/mL)	9.3	2.2	2.3
$AUC_{0-\infty}$ (μg × h/mL)	9.6	2.3	2.4
MRT (h)	4.6	4.2	5.4
T1/2 (h)	3.2	5.1	7.8
Tmax (h)	–	0.93	0.45
Cmax (μg/mL)	–	3.24	3.74
CL (mL/min/kg)	48	–	–
Vdss/kg (L/kg)	13.2	–	–

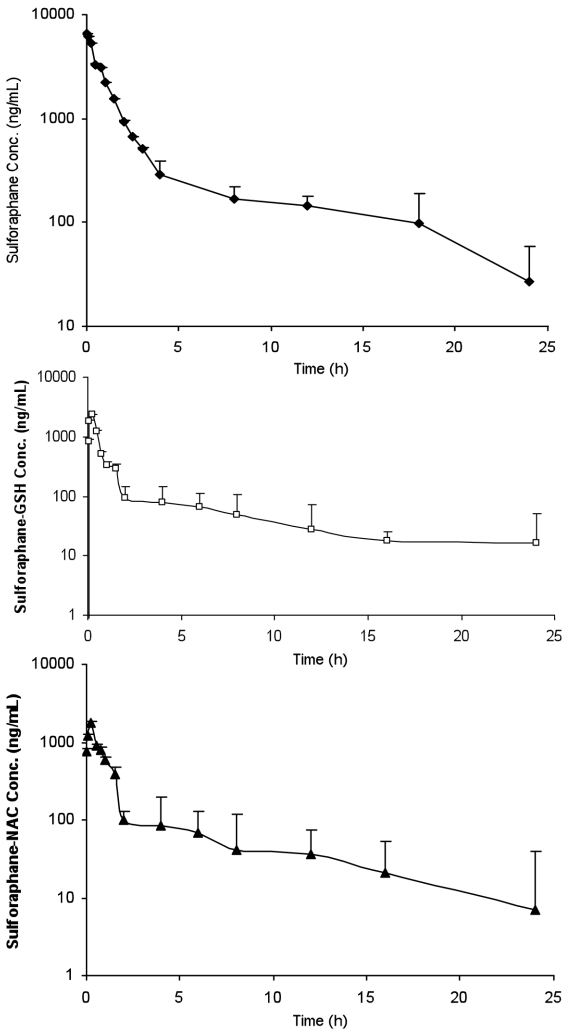

Figure 5. Concentration versus time profiles of (A) SFN and its major metabolites, (B) SFN-GSH, and (C) SFN-NAC in rat plasma, following intravenous bolus administration of SFN at a 25 mg/kg dose (*n* = 4). The data are presented as mean with standard error.

Conclusion

A simple and fast LC–MS–MS analytical method with high sensitivity was developed for the quantification of SFN and its metabolites in rat plasma. The method showed highly satisfactory accuracy and precision. Protein precipitation was used for sample preparation. A Develosil C_{30} column was used as the stationary phase. The method was successfully applied to study the pharmacokinetics of SFN in the rats, in which basic i.v. pharmacokinetic parameters such as clearance, terminal half-life, steady state volume of distribution were determined. The chromatographic conditions as well as sample preparation method of the current assay will likely facilitate the development and validation of LC–MS–MS analytical assay to analyze SFN in other biomatrices such as urine and tissue homogenates, which will be used in future subsequent studies.

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