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Simultaneous determination of sulforaphane and its major metabolites from biological matrices with liquid chromatography–tandem mass spectroscopy

Shruti Agrawal [a], Bozena Winnik [b], Brian Buckley [b], Lixin Mi [c], Fung-Lung Chung [c], Thomas J. Cook [a,b,*]

[a] *Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, United States*
[b] *Environmental and Occupational Health Sciences Institute, 170 Frelinghuysen Road, Piscataway, NJ 08854, United States*
[c] *Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, 3800 Reservoir Rd. LL Level, Washington, DC 20057, United States*

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Abstract

A simple, sensitive and specific LC–MS/MS method for the simultaneous determination of sulforaphane (SFN) and its major metabolites, the glutathione (SFN–GSH) and *N*-acetyl cysteine conjugates (SFN–NAC) from biological matrices was developed and validated. The assay procedure involved solid-phase extraction of all three analytes from rat intestinal perfusate using C2 extraction cartridges, whereas from rat plasma, metabolites were extracted by solid-phase extraction and SFN was extracted by liquid–liquid extraction with ethyl acetate. Chromatographic separation of SFN, SFN–GSH and SFN–NAC was achieved on a C8 reverse phase column with a mobile phase gradient (Mobile Phase A: 10 mM ammonium acetate buffer, pH: 4.5 and Mobile Phase B: acetonitrile with 0.1% formic acid) at a flow rate of 0.3 mL/min. The Finnigan LCQ LC–MS/MS was operated under the selective reaction monitoring mode using the electrospray ionization technique in positive mode. The nominal retention times for SFN–GSH, SFN–NAC and SFN were 8.4, 11.0 and 28.2 min, respectively. The method was linear for SFN and its metabolites with correlation coefficients >0.998 for all analytes. The limit of quantification was 0.01–0.1 μM depending on analyte and matrix, whereas the mean recoveries from spiked plasma and perfusate samples were approximately 90%. The method was further validated according to U.S. Food and Drug Administration guidance in terms of accuracy and precision. Stability of compounds was established in a battery of stability studies, i.e., bench-top, auto-sampler and long-term storage stability as well as freeze/thaw cycles. The utility of the assay was confirmed by the analysis of intestinal perfusate and plasma samples from single-pass intestinal perfusion studies with mesenteric vein cannulation in rats.

Keywords: Sulforaphane; LC–MS/MS; Validation; Cancer chemo-prevention

1. Introduction

Epidemiological data continue to support that dietary intake of cruciferous vegetables may reduce the risk of different types of malignancies [1–3]. The apparent cancer prevention effect of cruciferous vegetables is primarily attributed to isothiocyanates (ITCs) that occur naturally as the glucosinolate precursors in a variety of edible plants including watercress, broccoli, cabbage, Brussels sprouts, radish, and mustard [1–3]. Sulforaphane [SFN; 1-isothiocyanato-4-(methylsulfinyl)-butane], a naturally occurring member of the ITC family of cancer prevention agents, has received particular attention because of its potency [4–6]. This phytochemical is a potent inducer of the Phase 2 enzymes implicated in carcinogen detoxification and a competitive inhibitor of CYP2E1, which is involved in the activation of carcinogenic chemicals [7,8]. SFN is principally metabolized by the mercapturic acid pathway where an initial conjugation with glutathione promoted by glutathione transferases gives rise to the SFN–glutathione conjugate (SFN–GSH) that undergoes further enzymatic modification to produce the *N*-acetylcysteine conju-

* Corresponding author at: Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, United States. Tel.: +1 732 445 3831; fax: +1 732 445 3134.
E-mail address: tjcook@rci.rutgers.edu (T.J. Cook).

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gate (SFN–NAC). Thus SFN is metabolized to SFN–GSH and excreted in the form of SFN–NAC in the urine [9]. Despite increasing evidence of the efficacy of SFN in cancer prevention, our knowledge about the mechanisms of intestinal disposition, distribution, metabolism, and elimination of SFN and its metabolites is incomplete, partially due to the difficulty of developing specific and sensitive methods for measuring SFN and its metabolites in biological samples.

Several analytical methods based on high-pressure liquid chromatography (HPLC) coupled to UV or mass detection have been described for the analyses of SFN from plant extracts or from urine [10–17] and the goal of the study described herein is to improve on these methods by reducing analytical time, simplifying extraction procedures, adapting the methods to analyzing constituents in plasma, and to increase extraction recoveries. To increase the spectroscopic sensitivity of the assay, researchers have used a cyclocondensation reaction in which vicinal sulfhydryl groups of 1,2-benzenedithiol react quantitatively with ITCs to produce a cyclic condensation product, 1,3-benzodithiole-2-thione [13,14]. Recently, this method has been modified for the analysis of ITCs in human plasma [15,16]. However, this method measures the total concentration of ITCs in the blood and does not distinguish between parent compounds and their metabolites. As for MS-based assays, Vermeulen et al. have reported the analysis of ITC mercapturic acids in urine by LC–MS/MS [17]. The extraction method, however, was only applied for the extraction of polar metabolites from urine. In addition, the run time employed was 55 min. Recently, LC–MS method for the analysis of SFN from plasma has been reported by Hu et al. [18], where the samples were prepared by centrifugal filtration.

Thus, several bioanalytical methods exist for the quantification of SFN from urine and plasma or its metabolites from urine based on spectroscopic or mass detection. No sensitive method exists, however, for the simultaneous detection of SFN and its metabolites from plasma or serum samples. The development of an assay for the measurement of SFN and its metabolites in plasma poses an inherent challenge of extracting these analytes having wide differences in polarity coupled with the plasma protein binding. Hence, the objective of this investigation was to develop a specific and sensitive LC–MS/MS assay for SFN and its major metabolites from biological matrices such as intestinal perfusate and plasma. The described method was validated in terms of selectivity, linearity, limit of quantification, accuracy, precision, and stability based on U.S. Food and Drug Administration (FDA) guidelines [19] and successfully applied to the analysis of perfusate and plasma samples from single-pass intestinal perfusion studies with mesenteric vein cannulation in Sprague-Dawley rats.

2. Experimental

2.1. Chemicals and reagents

SFN (purity >99%) was purchased from LKT Laboratories (St. Paul, MN). SFN–GSH and SFN–NAC (>95% purity) were synthesized by Dr. Chung and group, Georgetown University Medical Center (Washington, DC). Acetonitrile, ethyl acetate, methanol of HPLC grade were obtained from Mallinckrodt Baker (Phillipsburg, NJ). Formic acid of mass-spectroscopic grade and all other reagents were purchased from Sigma–Aldrich (St. Louis, MO). All aqueous solutions including buffer for the HPLC mobile phase were prepared with MilliQ (Millipore, Milford, MA) grade water. The control rat plasma was purchased from Hilltop Lab Animals (Scottdale, PA).

2.2. Equipment

For solid-phase extraction a Waters extraction manifold (Milford, MA) along with Bond-elut-C2 500 mg, 3 mL extraction cartridges (Varian Inc., Walnut Creek, CA) were used. Samples were analyzed on an HPLC system consisting of a Waters 2690 LC separation module coupled to a Waters 996 photodiode array (PDA) UV detector and a Thermo-Finnigan LCQ Classic detector (San Jose, CA) fitted with an ESI interface. Analytical column used was SymmetryShield 8, 4.6 mm × 250 mm, 5 μm (Waters, Milford, MA) connected to C8 guard cartridge. Other instruments used included an electronic balance AG-245 (Greifensee, Switzerland), Branson 3210 sonicator (The Hague, The Netherlands), Biofuge primo from Heraeus (Hanau, Germany), and Vortex Genie 2 and Finnpipettes from Fisher Scientific (Pittsburgh, PA).

2.3. Preparation of standards and quality control samples

Primary stock solutions of SFN and SFN–NAC were prepared in acetonitrile whereas a stock solution of SFN–GSH was prepared in acetonitrile:water (50:50) with 0.1% formic acid. The stock solutions were further diluted quantitatively with acetonitrile to give working stock solutions of various concentrations for the preparation of calibration and quality control (QC) samples and were stored at −80 °C. A calibration (standard) curve and QC samples were prepared by adding 10 μL of standard solution to 990 μL of biological matrix. Calibration curve samples prepared daily were composed of a blank sample and seven standards in the concentration range of 0.01–10 μM for SFN, 0.05–10 μM for SFN–GSH and 0.02–10 μM for SFN–NAC from intestinal perfusate; and 0.02–10 μM for SFN, 0.1–10 μM for SFN–GSH and 0.05–10 μM for SFN–NAC from plasma. Samples for the determination of recovery, precision and accuracy were prepared by spiking blank intestinal perfusate or blank plasma in bulk at appropriate concentrations, aliquoted into different tubes and, depending on the nature of experiment, samples were stored at −80 °C until analysis.

2.4. Sample preparation procedure

From intestinal perfusate all the three analytes, i.e., SFN, SFN–GSH and SFN–NAC were isolated by solid-phase extraction using C2 extraction cartridges. A 500 μL perfusate was acidified with 100 μL of 3% formic acid and mixed for 15 s on a cyclomixer. Extraction cartridges were preconditioned with 2 mL methanol followed by 2 mL 0.1% formic acid. After application of sample, cartridges were washed with 2 mL

Table 1
Gradient mobile phase condition for the analysis of SFN and its metabolites

Time (min)	Flow (μL/min)	% A	% B
0	300	80	20
12	300	80	20
20	300	25	75
30	300	25	75
32	300	80	20
35	300	80	20

A: 10 mM ammonium acetate buffer pH 4.5; B: Acetonitrile with 0.1% formic acid.

water:methanol (95:5). The elution step consisted of 1 mL of methanol followed by 1 mL of acetonitrile. The combined eluent was evaporated by gentle stream of nitrogen at 50 °C and reconstituted in a 150 μL of mixture of ammonium acetate buffer (10 mM, pH 4.5) and acetonitrile with 0.1% formic acid (50:50).

From plasma, the metabolites were extracted by the solid-phase extraction mentioned above for the perfusate except the volume of plasma used was 100 μL. SFN from plasma (100 μL) was extracted by liquid–liquid extraction with ethyl acetate. Plasma aliquot was taken into 2 mL Eppendorf tubes, and extracted with 1.5 mL of ethyl acetate by vortexing for 2 min. After centrifugation at 5000 rpm for 5 min, the organic layer was separated and evaporated under nitrogen at 50 °C. The residue obtained was reconstituted in a 150 μL of mixture of ammonium acetate buffer (10 mM, pH 4.5) and acetonitrile with 0.1% formic acid (50:50) and transferred into HPLC vial for LC–MS/MS analysis.

2.5. Chromatographic and mass spectroscopic conditions

For the determination of suitable chromatographic conditions, different gradient mobile phases of water with 0.1% formic acid or an ammonium acetate buffer and acetonitrile with 0.1% formic acid were tested on C18 and C8 columns. The optimized method used a binary gradient mobile phase with 10 mM ammonium acetate buffer (pH 4.5) as mobile phase A and acetonitrile with 0.1% formic acid as mobile phase B (Table 1) at the flow rate of 0.3 mL/min. The sample extracts (75 μL) were eluted on a C8 column (SymmetryShield RP8, 4.6 mm × 250 mm, 5.0 μm) with a total run time of 35 min. All the chromatographic measurements were performed at room temperature and the autosampler was maintained at 4 °C.

The quantitation was achieved by MS/MS detection in positive ion mode for all the three analytes. The LC flow was introduced into the ESI interface following detection by UV absorption measured from 200 to 400 nm. High-purity (99% pure) nitrogen gas served both as sheath gas with an operating pressure of 90 units and as auxiliary gas with a flow rate of 4.0 units, respectively. The heated capillary temperature and spray voltage were maintained at 275 °C and 7 kV, respectively. In the full scan mode, the mass spectrometer was operated over a range of *m/z* 100–500 in the centroid mode, 1 microscan, and automatic gain control on, and inject waveform off. Detection of the ions was performed in selected reaction monitoring mode with the collision energy of 30% monitoring the transition of *m/z* 178 precursor ion to *m/z* 114 product ion for SFN, *m/z* 485 precursor ion to *m/z* 356 product ion for SFN–GSH and *m/z* 341 precursor ion to *m/z* 178 product ion for SFN–NAC. A solution of caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate, and Ultramark 1621 prepared in a mixture of acetonitrile:methanol:water (2:1:1) containing 1% acetic acid was used for calibration. The entire system, excluding the MS detector, was controlled using Waters Millennium Software (Ver. 3.2) while the MS detector was controlled by the Xcalibur software (Thermo Finnigan Corp., San Jose, CA).

2.6. Method validation

Analytical method validation was performed in accordance to the recommendations published by the FDA [19]. The validation parameters studied were selectivity, specificity, limit of detection, lowest limit of quantitation, linearity and range, accuracy and precision, extraction recoveries and stability assays.

2.6.1. Specificity and selectivity

The lack of chromatographic interference from endogenous perfusate or plasma components was investigated by comparing chromatograms of blank and spiked samples. Limit of detection and lowest limit of quantitation were determined based on signal to noise ratio of 3 and 10, respectively, detected from spiked and blank samples. For the determination of matrix effect, standard compounds were spiked to blank extracts of perfusate and plasma and the responses were compared to those of non-extracted standards.

2.6.2. Calibration curve

Calibration curves were acquired by plotting the peak area against the nominal concentration of calibration standards. The concentrations used were in the range of 0.01–10 μM for SFN, 0.05–10 μM for SFN–GSH and 0.02–10 μM for SFN–NAC from perfusate, whereas 0.02–10 μM for SFN, 0.1–10 μM for SFN–GSH and 0.05–10 μM for SFN–NAC from plasma. The results were fitted to linear regression analysis using $1/\chi^2$ as weighting factor. The minimally acceptable correlation coefficient (r^2) for the calibration curve was 0.99 or greater. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value except at the lowest limit of quantitation, which was set at ±20% [19,20].

2.6.3. Precision and accuracy

The intra-day precision and accuracy were estimated by analyzing four replicates containing SFN and metabolites at four different QC levels, i.e., 0.01, 0.03, 3.0 and 8.0 μM for SFN, 0.05, 0.2, 3.0 and 8.0 μM for SFN–GSH, 0.02, 0.06, 3.0 and 8.0 μM for SFN–NAC from perfusate whereas 0.02, 0.06, 3.0 and 8.0 μM for SFN, 0.1, 0.3, 3.0 and 8.0 μM for SFN–GSH, 0.05, 0.2, 3.0 and 8.0 μM for SFN–NAC from plasma. The inter-assay precision was determined by analyzing four level QC samples on four different runs. Accuracy was calculated on the basis of quotient of the averaged measurements and the nominal value and expressed in percent. The criteria for acceptability of data included accuracy within ±15% deviation from the nom-

inal values and a precision of within ±15% relative standard deviation (R.S.D.), except for the lowest limit of quantitation, where it should not exceed 20% of R.S.D.

2.6.4. Extraction recovery

The extraction recoveries of SFN and metabolites from perfusate or plasma were determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 4$) with the response of analytes from non-extracted standard solutions at equivalent concentrations.

2.6.5. Stability experiments

The in-autosampler stability of SFN and metabolites in the injection solvent was determined periodically by injecting replicate preparation of processed samples up to 28 h at 4 °C after initial injection. The peak areas obtained at initial cycle were used as reference to determine the relative stability of the analytes at subsequent points. Bench-top stability in the biological matrices during an 8 h period was determined at ambient temperature at four concentrations, in triplicate. Freezer stability of SFN and metabolites in perfusate as well as in plasma was assessed by analyzing QC samples stored at −80 °C for one month. The stability of analytes in both the matrices was also checked after repeated freeze/thaw cycles. The samples were stored at −80 °C between freeze/thaw cycles. The samples were processed using the same procedure as described in the sample preparation section. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ±15% deviation) and precision (i.e., ±15% R.S.D.), except for the lowest limit of quantitation, where it should not exceed 20% of deviation for accuracy and 20% of R.S.D. for precision.

2.7. Permeability estimation of SFN in rat ileum

The applicability of the developed LC–MS/MS method was tested by quantitating the concentrations of SFN, SFN–GSH, and SFN–NAC in mesenteric vein blood and intestinal perfusate samples obtained from in situ single-pass intestinal perfusion experiments in the rat ileum using a method based on the report by Johnson et al. [21]. In brief, Sprague Dawley rats weighing 250–300 g and pre-cannulated at the jugular vein were obtained from Hilltop Lab Animals. After anesthetizing with ketamine and xylazine, rats were placed on a heating pad. Two additional sites were cannulated: ileal segment and mesenteric vein draining the segment. Two notched Teflon cannulae were placed at the ends of ileal segment and perfused with 10 μM of SFN in iso-osmolar ammonium acetate buffer at the flow rate of 0.2 mL/min. The mesenteric vein draining the ileal segment was then immediately cannulated with a 24 G Angeocath catheter and then connected to silastic tubing. Whole blood obtained from donor rats was infused into the experimental rat via the jugular vein cannula. The outlet perfusate samples and the mesenteric blood samples were collected at 5 min intervals. The apparent in situ permeability coefficients (Fig. 3) were calculated from the perfusate (P_e) and plasma concentrations (P_b) as previously described by Singhal et al. [22].

3. Results and discussion

3.1. Method development, specificity and selectivity

Initially, for the simultaneous extraction of SFN, SFN–GSH and SFN–NAC from plasma, different solvents including methanol, acetonitrile, ethyl acetate, dichloromethane were tried. The polar solvents were found to extract other polar components from plasma and gave a very high background noise in the mass detector. In case of perfusate, only water immiscible non-polar solvents like ethyl acetate and dichloromethane could be used for liquid–liquid extraction. In non-polar solvents, the extraction recoveries of polar metabolites were very low. Therefore, we tried solid-phase extraction technique to improve the recovery of metabolites from the perfusate and plasma. Of the various cartridges used, the 500 mg C2 cartridges improved recoveries of metabolites from perfusate as well as from plasma. The elution step was optimized by using 1 mL of methanol followed by 1 mL of acetonitrile. To further improve the recovery of analytes, samples were acidified with 3% formic acid and cartridges were preconditioned with 0.1% formic acid. This has resulted in 90% extraction recovery of all the three analytes from perfusate and both the metabolites from plasma compared to standard solutions at equivalent concentrations. However, with solid-phase extraction, recoveries of SFN from plasma was very low and inconsistent. Therefore, for the extraction of SFN from plasma, a separate liquid–liquid extraction step with ethyl acetate was used that had achieved an extraction recovery of 88%. The investigation of the matrix effect was done by post-extraction spiking. During the optimization of the extraction process, the matrix effect was observed with methanol and acetonitrile extracts. However, after using solid-phase extraction for the metabolites and ethyl acetate extraction for SFN, the matrix effect was negligible. This is also evident from the approximate 90% recovery of all the analytes compared to non-extracted standards. In addition, during the optimization steps, it was observed

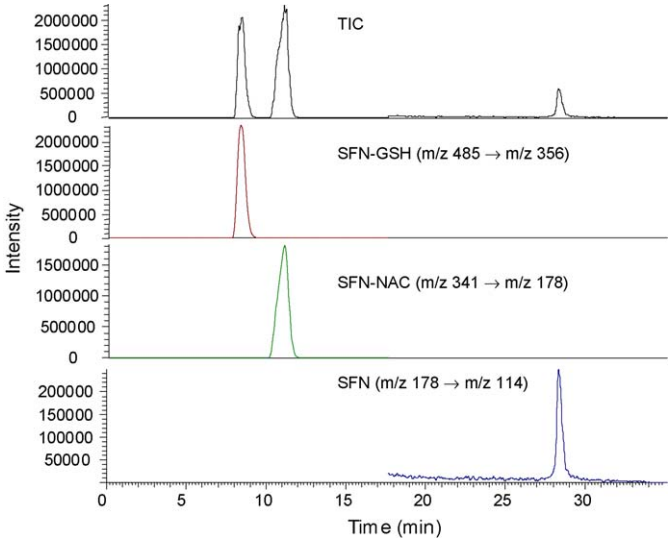

Fig. 1. Representative LC–MS/MS chromatograms of SFN and its metabolites in rat intestinal perfusate.

that SFN binds to glass and results in low recovery with the use of glassware. Hence, use of glassware including HPLC injection vials was avoided in all the experiments.

To achieve balanced results in terms of peak shape and sensitivity of all the components, detailed efforts in the evaluation of various mobile phases was undertaken. For better resolution in all the three analytes, various gradient mobile phase conditions were tried such as water with 0.1% formic acid, acetonitrile with 0.1% formic acid, and ammonium acetate buffer of various molarities and pH. SFN was always well resolved from the metabolites, whereas changes in pH of the mobile phase affected the ionization of the metabolites as well as the resolution between SFN–GSH and SFN–NAC. Both the metabolites were co-eluting when water with 0.1% formic acid was used as one of the component of the mobile phase. The optimal separation and ionization of all the components were achieved with ammonium acetate buffer. The final mobile phase composition at which the separation of all the three analytes was achieved is shown in Table 1. For the quantitative detection by mass spectroscopy, selective reaction monitoring mode was used for the simultaneous estimation of SFN and its metabolites since it is a powerful analytical technique for pharmacokinetic studies and provides selectivity, sensitivity and specificity requirements for analytical methods. By the use of selective reaction monitoring, a key capability of tandem mass spectrometers, the selection and quantification of compound-specific ion-pairs enable a reduction in the interference of co-eluting substances and a considerable improvement in assay selectivity. The matrix effect on the present method was evaluated by spiking blank perfusate or plasma extracts across the linearity range and the results were compared with pure samples having similar concentrations. It was found that there was no significant difference for peak responses between these samples indicating minimal matrix effect. Carry-over in the LC–MS/MS analysis was evaluated

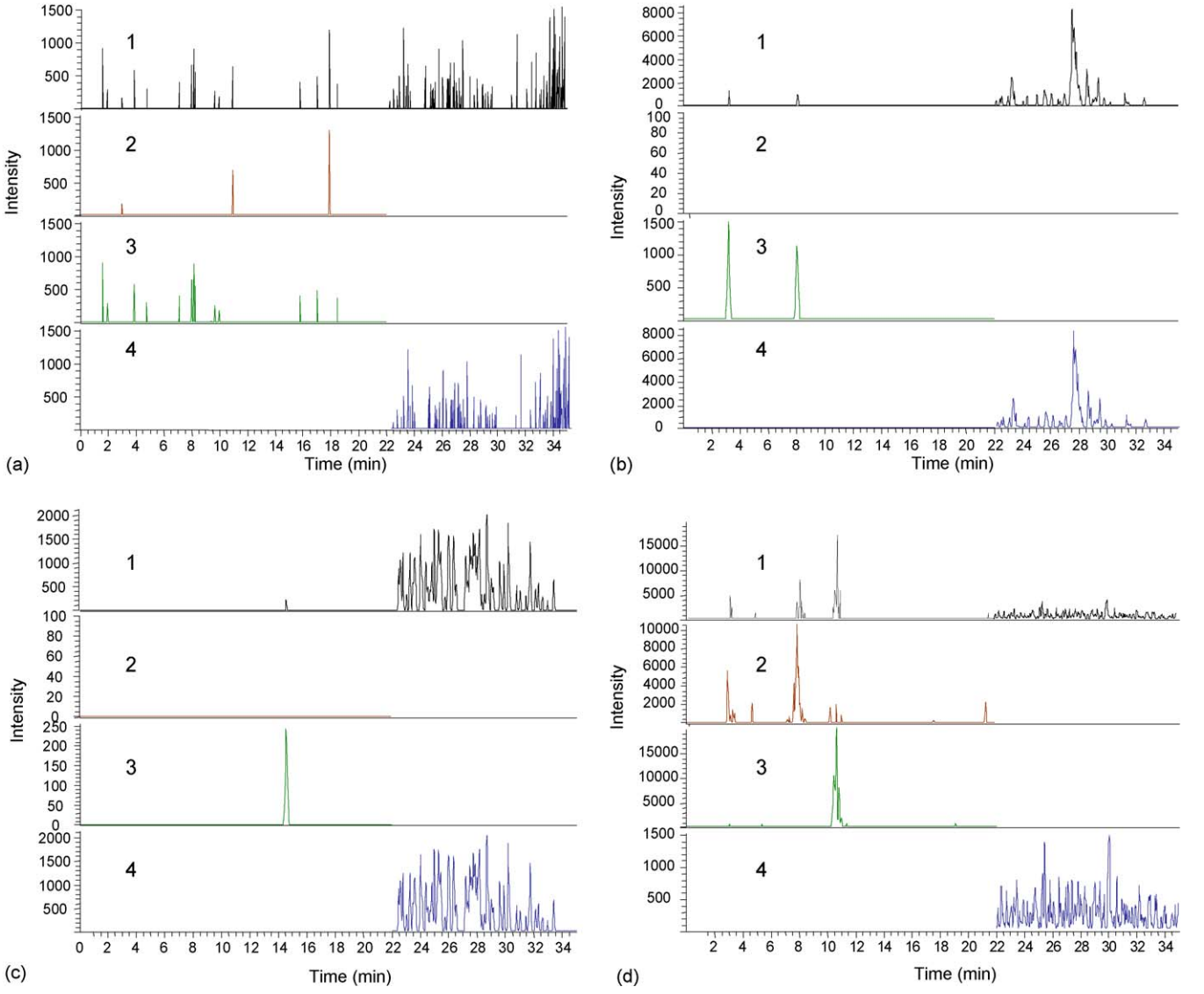

Fig. 2. Chromatograms representing (a) blank plasma sample after ethyl acetate extraction, (b) ethyl acetate extract at the lowest limit of quantitation to detect SFN, (c) blank plasma sample after solid-phase extraction and (d) solid-phase extraction sample at the lowest limit of quantitation to detect SFN–GSH and SFN–NAC conjugates. Chromatogram 1: TIC, 2: SFN–GSH (m/z 485 → m/z 356), 3: SFN–NAC (m/z 341 → m/z 178) and 4: SFN (m/z 178 → m/z 114).

daily by checking blank and control samples and no significant interference from previous injections was observed. While establishing the method, care was taken in optimizing declustering potential and collision energy to obtain high sensitivity for all the three components. The selective reaction monitoring parameters were fully optimized for SFN and conjugates to increase the sensitivity and reduce the artifacts from biological samples. In addition to the major fragment at 356 *m/z*, the GSH conjugate fragments to the parent compound, SFN, producing *m/z* of 178. The selective reaction monitoring of NAC conjugate is done at 341 *m/z* to 178 *m/z*. Subsequently, a run time of 35 min was employed in order to achieve optimal baseline resolution of the two hydrophilic metabolites for accurate quantitation without interference. The retention times of SFN–GSH, SFN–NAC and SFN were 8.4, 11.0 and 28.2 min, respectively (Fig. 1). These optimized conditions of sample pretreatment, liquid chromatographic and mass detection parameters enabled the establishment of the lowest limit of quantitation as low as 0.01 μM for SFN and also provide for the simultaneous detection of SFN and its major metabolites in a run-time of 35 min. Fig. 2 represents chromatographs of SFN and its metabolites from plasma after solid-phase and ethyl acetate extraction along with respective blank samples.

3.2. Method validation

The above developed LC–MS/MS method for detection of SFN and its major metabolites was validated according to USFDA guidance [19]. Validation parameters for all the components from perfusate are summarized in Table 2, whereas from plasma are summarized in Table 3. The calibration curve was constructed using seven calibration samples in the range of 0.01–10 μM for SFN, 0.05–10 μM for SFN–GSH and 0.02–10 μM for SFN–NAC from perfusate, whereas 0.02–10 μM for SFN, 0.1–10 μM for SFN–GSH and 0.05–10 μM for SFN–NAC from plasma. The standard curve had a reliable reproducibility over the standard concentrations of all the analytes across the calibration range. Calibration curves were prepared by determining the best fit of peak area ratios verses concentration and fitted to linear regression using weighing factor ($1/\chi^2$). The average regression was always more than 0.998 from both the matrices. The R.S.D. at the lowest limit of quantitation was 7.3–11.6% for all the analytes. The calibration curve ranges were selected based on concentrations found in published pharmacokinetic studies [18].

The detailed accuracy and precision data for intra- and inter-day test samples are presented in Tables 2 and 3. The intra-day accuracy (%) at the lowest limit of quantitation for SFN, SFN–GSH and SFN–NAC from perfusate ranged from 90.2 to 110.6, 83.7 to 118.9 and 86.5 to 114.2, respectively whereas in plasma intra-day accuracy ranged from 88.6 to 116.8, 82.8 to 114.9 and 83.7 to 114.7, respectively. The intra-day accuracies at higher QC samples were in the range of 85.3 to 115.3 from perfusate and 86.8 to 112.4 from plasma, respectively. The inter-day accuracies (% deviation) were also within ±20% at the lowest limit of quantitation and ±15% at remaining test

Table 2
Validation summary of SFN and its metabolites from rat intestinal perfusate

Validation parameter	SFN	SFN–GSH	SFN–NAC
Linearity			
Calibration range	0.01–10 μM	0.05–10 μM	0.02–10 μM
Correlation	0.999	0.998	0.999
Limit of detection	0.003 μM	0.01 μM	0.007 μM
Lowest limit of quantitation	0.01 μM	0.05 μM	0.02 μM
Intra-day accuracy (% bias)			
Lowest limit of quantitation	−9.8 to 10.6	−16.3 to 18.9	−13.5 to 14.2
Quality control (low)	−10.0 to 9.4	−14.7 to 11.6	−12.1 to 13.7
Quality control (medium)	−7.8 to 8.9	−12.0 to 15.3	−8.4 to 5.8
Quality control (high)	−7.4 to 3.6	−8.3 to 4.9	−6.1 to 6.3
Inter-day accuracy (% bias)			
Lowest limit of quantitation	−10.9 to 11.2	−17.1 to 16.8	−15.9 to 16.7
Quality control (low)	−12.3 to 10.4	−13.2 to 14.6	−10.7 to 9.5
Quality control (medium)	−6.9 to 8.1	−9.8 to 6.3	−7.6 to 6.9
Quality control (high)	−6.2 to 4.9	−7.7 to 5.4	−5.9 to 8.6
Intra-day precision (% CV)			
Lowest limit of quantitation	11.4	10.3	8.6
Quality control (low)	6.0	2.3	2.5
Quality control (medium)	0.5	1.9	1.3
Quality control (high)	1.7	1.1	0.9
Inter-day precision (% CV)			
Lowest limit of quantitation	10.8	14.3	7.6
Quality control (low)	5.7	5.2	5.0
Quality control (medium)	1.4	3.7	2.2
Quality control (high)	1.5	3.9	5.8
Extraction recovery (%)	92.6	89.8	91.2

Table 3
Validation summary of SFN and its metabolites from rat plasma

Validation parameter	SFN	SFN–GSH	SFN–NAC
Linearity			
Calibration range	0.02–10 μM	0.1–10 μM	0.05–10 μM
Correlation	0.998	0.998	0.998
Limit of detection	0.005 μM	0.03 μM	0.005 μM
Lowest limit of quantitation	0.02 μM	0.1 μM	0.05 μM
Intra-day accuracy (% bias)			
Lowest limit of quantitation	−11.4 to 16.8	−17.2 to 14.9	−16.3 to 14.7
Quality control (low)	−13.2 to 8.6	−9.9 to 10.3	−11.6 to 12.4
Quality control (medium)	−8.1 to 7.5	−10.3 to 6.8	−9.1 to 8.8
Quality control (high)	−6.7 to 4.3	−8.9 to 5.6	−7.5 to 6.7
Inter-day accuracy (% bias)			
Lowest limit of quantitation	−13.5 to 15.2	−13.4 to 8.5	−11.0 to 13.1
Quality control (low)	−7.7 to 1.6	−4.2 to 4.9	−9.6 to 8.8
Quality control (medium)	−2.7 to 2.3	−5.3 to 8.6	−6.3 to 4.7
Quality control (high)	−3.4 to 1.4	−7.1 to 4.3	−2.6 to 3.9
Intra-day precision (% CV)			
Lowest limit of quantitation	8.3	11.6	7.3
Quality control (low)	3.1	2.9	4.6
Quality control (medium)	1.4	3.2	2.4
Quality control (high)	1.1	1.7	1.6
Inter-day precision (% CV)			
Lowest limit of quantitation	10.4	8.7	7.9
Quality control (low)	6.0	8.1	5.3
Quality control (medium)	1.3	1.9	1.4
Quality control (high)	2.8	2.3	2.3
Extraction recovery (%)	88.3	90.5	89.7

concentrations for all the analytes and from both the matrices. The intra-day precision (R.S.D.) at the lowest limit of quantitation from perfusate were 11.4, 10.3 and 8.6 for SFN, SFN–GSH and SFN–NAC, respectively, whereas the corresponding values from plasma were 8.3, 11.6 and 7.3, respectively. The % R.S.D. for intra-day sample analysis for the higher concentration QC samples were in the range of 0.9–6.0 from perfusate and 1.1–4.6 from plasma. The inter-day precision was also within the acceptable range of 20% at the lowest limit of quantitation and 15% at other QC concentrations.

Extraction recovery was evaluated by comparison of the mass ion peak areas of the extracted samples at four QC levels with

Table 4
Stability data of SFN and its metabolites from rat intestinal perfusate

QC level	Stability condition	% Remaining ± S.D.		
		SFN	SFN–GSH	SFN–NAC
Lowest limit of quantitation	3 freeze-thaw cycles	95.6 ± 9.9	87.2 ± 13.9	103.1 ± 8.3
	8 h bench-top	91.4 ± 13.2	68.3 ± 14.5	99.7 ± 10.4
	28 h in-injector	94.5 ± 9.7	92.8 ± 11.6	98.6 ± 7.8
	30 days at −80 °C	97.1 ± 10.8	89.7 ± 13.4	102.9 ± 8.5
Quality control (low)	3 freeze-thaw cycles	98.2 ± 7.6	90.3 ± 9.2	108.6 ± 6.0
	8 h bench-top	90.1 ± 8.8	74.2 ± 10.7	101.3 ± 3.9
	28 h in-injector	96.3 ± 7.9	93.9 ± 13.6	103.8 ± 9.7
	30 days at −80 °C	94.6 ± 11.5	92.8 ± 8.4	98.5 ± 5.1
Quality control (medium)	3 freeze-thaw cycles	94.3 ± 9.0	88.4 ± 11.9	96.2 ± 8.8
	8 h bench-top	92.6 ± 4.8	73.9 ± 9.3	99.4 ± 5.7
	28 h in-injector	101.5 ± .9.2	91.8 ± 10.2	101.9 ± 4.1
	30 days at −80 °C	97.4 ± 7.1	95.1 ± 6.7	97.7 ± 6.0
Quality control (high)	3 freeze-thaw cycles	90.9 ± 11.5	94.3 ± 8.4	103.1 ± 2.5
	8 h bench-top	93.4 ± 6.4	77.2 ± 10.7	96.6 ± 3.2
	28 h in-injector	96.1 ± 7.9	95.4 ± 9.8	99.4 ± 6.4
	30 days at −80 °C	92.6 ± 5.8	90.0 ± 7.3	105.9 ± 8.1

Table 5
Stability data of SFN and its metabolites from rat plasma

QC level	Stability condition	% Remaining ± S.D.		
		SFN	SFN–GSH	SFN–NAC
Lowest limit of quantitation	3 freeze-thaw cycles	98.0 ± 10.4	91.6 ± 14.4	98.5 ± 13.1
	8 h bench-top	93.7 ± 8.9	72.8 ± 11.5	105.3 ± 7.4
	28 h in-injector	96.3 ± 7.6	96.3 ± 8.1	102.6 ± 8.2
	30 days at −80 °C	104.8 ± 13.2	97.4 ± 12.5	100.9 ± 10.5
Quality control (Low)	3 freeze-thaw cycles	96.4 ± 8.3	93.7 ± 6.0	101.6 ± 7.2
	8 h bench-top	89.7 ± 7.5	76.9 ± 9.3	96.5 ± 8.6
	28 h in-injector	94.1 ± 6.5	98.5 ± 11.1	106.1 ± 9.7
	30 days at −80 °C	97.8 ± 9.2	94.6 ± 8.4	99.2 ± 8.8
Quality control (Medium)	3 freeze-thaw cycles	97.6 ± 5.9	94.9 ± 8.7	104.5 ± 3.9
	8 h bench-top	91.8 ± 6.4	74.2 ± 9.1	96.3 ± 5.2
	28 h in-injector	99.2 ± 4.5	94.6 ± 7.2	105.5 ± 6.8
	30 days at −80 °C	100.6 ± 7.3	98.3 ± 5.0	104.8 ± 8.7
Quality control (High)	3 freeze-thaw cycles	92.7 ± 6.4	95.4 ± 6.9	93.4 ± 4.1
	8 h bench-top	91.5 ± 4.3	80.2 ± 9.4	101.8 ± 5.7
	28 h in-injector	97.8 ± 5.9	97.3 ± 7.6	103.6 ± 2.9
	30 days at −80 °C	95.6 ± 8.5	96.9 ± 4.5	97.4 ± 8.4

the standard solutions of equivalent concentrations. The mean extraction recovery of SFN, SFN–GSH and SFN–NAC from perfusate was 92.6, 89.8 and 91.2%, respectively whereas from plasma it was 88.3, 90.5 and 89.7%.

The stability data of SFN and its conjugates under various conditions from perfusate is summarized in Table 4. Stability studies were conducted at four QC levels. All the three analytes were stable after three freeze/thaw cycles, in autosampler for 28 h and long-term storage at −80 °C for one month. In these stability conditions, the samples deviated within the nominal concentrations and the results were found to be within the assay variables. In bench-top stability at ambient temperature for 8 h, SFN and SFN–NAC were found to be stable; however, SFN–GSH was unstable and found to degrade up to 25–30% at room temp. Similar results were also obtained for stability studies from plasma (Table 5). It is recommended that the sample processing involving SFN–GSH should be done over ice to avoid the loss of drug due to degradation at the higher temperature.

3.3. Application of the developed LC–MS/MS method

To begin to investigate the intestinal disposition of SFN in rats, the developed LC–MS/MS method was successfully applied to the determination of the intestinal permeability values (Fig. 3) of SFN, SFN–GSH and SFN–NAC from single-pass intestinal perfusion studies with mesenteric blood sampling. In those studies, only SFN was perfused in the intestine, therefore, first-pass intestinal metabolism would be indicated by the appearance of SFN–GSH or SFN–NAC in the mesenteric blood. The perfusion studies showed that SFN was well absorbed from the ileum with an average $P_{e(SFN)}$ value of 2.73×10^{-4} cm/s based on the disappearance of parent compound from the perfusate. However, the appearance of SFN in the mesenteric blood was very low ($P_{b(SFN)} = 7.09 \times 10^{-6}$ cm/s), while the permeability based on the appearance of the glutathione conjugate ($P_{b(SFN–GSH)}$) was as high as 1.96×10^{-4} cm/s. The rate of formation of the NAC conjugate was very low ($P_{b(SFN–NAC)} = 6.13 \times 10^{-7}$ cm/s). The predominant appearance of SFN–GSH in the mesenteric blood after perfusion of SFN

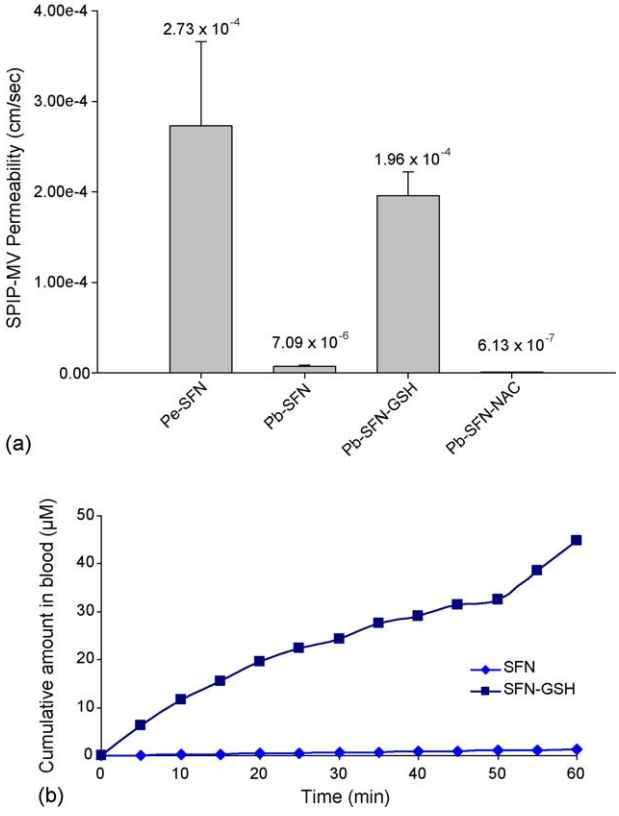

Fig. 3. (a) Permeability values of SFN, SFN–GSH and SFN–NAC calculated from intestinal perfusate and from mesenteric blood obtained from single-pass intestinal perfusion experiments ($n = 4$, mean ± standard deviation), (b) Representative plot of the cumulative amount of SFN and SFN–GSH appearing in mesenteric blood.

suggests substantial first-pass intestinal metabolism of SFN within the enterocytes or rapid conjugation in the blood based on its chemical reactivity. The intestinal disposition of SFN and its rate and form of appearance in the blood may provide better insight into its activity as a cancer prevention agent and to developing optimized dosing strategies for prevention modalities.

4. Conclusions

A simple, highly sensitive and validated LC–MS/MS method has been developed for the simultaneous analysis of SFN and its metabolites from biological matrices. Compared to the previously described assays, this method provides superior sensitivity with the lowest limit of quantitation as low as 0.01 μM for SFN. The developed method was successfully applied for analysis of perfusate and plasma samples to study the intestinal disposition of SFN from rat ileum.

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