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Quantitative measurement of sulforaphane, iberin and their mercapturic acid pathway metabolites in human plasma and urine using liquid chromatography–tandem electrospray ionisation mass spectrometry

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Abstract

A quantitative liquid chromatography positive ion electrospray tandem mass spectrometric method for the simultaneous determination of sulforaphane, iberin and their metabolites in human urine and plasma is described. The stability of the metabolites was determined in aqueous solution and in human plasma. Gradient liquid chromatographic separation was performed on a Zorbax SB-Aq 3.5 μm (100 × 2.1 mm) column, using a mobile phase (flow rate 0.25 mL/min) consisting of ammonium acetate buffer at pH 4 and acetonitrile. Butyl thiocarbamoyl L-cysteine was used as internal standard. The assay was linear ($r^2 > 0.99$) over the range of 0.03–300 μM in urine and 0.03–15 μM in plasma with intra- and inter-day assay precision (<10% CV) and accuracy (<20%). The lower limits of quantitation were in the range of 10–150 nmol/L. The method has been used to report, for the first time, individual quantitative measurement of each of the mercapturic acid pathway metabolites of sulforaphane and iberin in both human plasma and urine following a dietary study of broccoli consumption.

Keywords: Isothiocynates; Glucosinolates; Sulforaphane; Iberin; Electrospray ionisation mass spectrometry; Liquid chromatography; Metabolism; Mercapturic acid pathway; Plasma; Urine

1. Introduction

The high dietary content of cruciferous vegetables such as broccoli has been associated with a reduction in cancer risk by epidemiological studies [1–4]. Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)-butane) and iberin (1-isothiocyanato-3-(methylsulfinyl)-propane) are the major isothiocyanates (ITCs) in broccoli, derived from methylsulphinyl glucosinolates which are found in high concentrations in the florets of the plant [5]. These glucosinolates are converted to ITCs by plant thioglucosidases or by microbial metabolism in the colon [5]. Sulforaphane and iberin have been shown to have direct effects in vitro on phase I and phase II metabolising enzymes [6–8], on inducers of cell cycle arrest and on apoptosis [9–10] and consequently are viewed as candidates to explain the mechanism of the anti-cancer action of crucifer consumption. ITCs are absorbed rapidly into the body and are then conjugated with glutathione and metabolised by the mercapturic acid pathway and finally excreted in urine, predominantly as *N*-acetyl cysteine conjugates [5] (Fig. 1). However, the biokinetics of these ITCs and their metabolites are poorly understood since there is little data available on their absorption, distribution and excretion in humans. The main reason for this absence of data is that there has been no analytical method capable of measuring the individual ITCs and their metabolites in low concentrations in biofluids such as plasma and urine. This paper addresses the issues which have restricted the availability of analytical methods for these compounds and describes two new methods for

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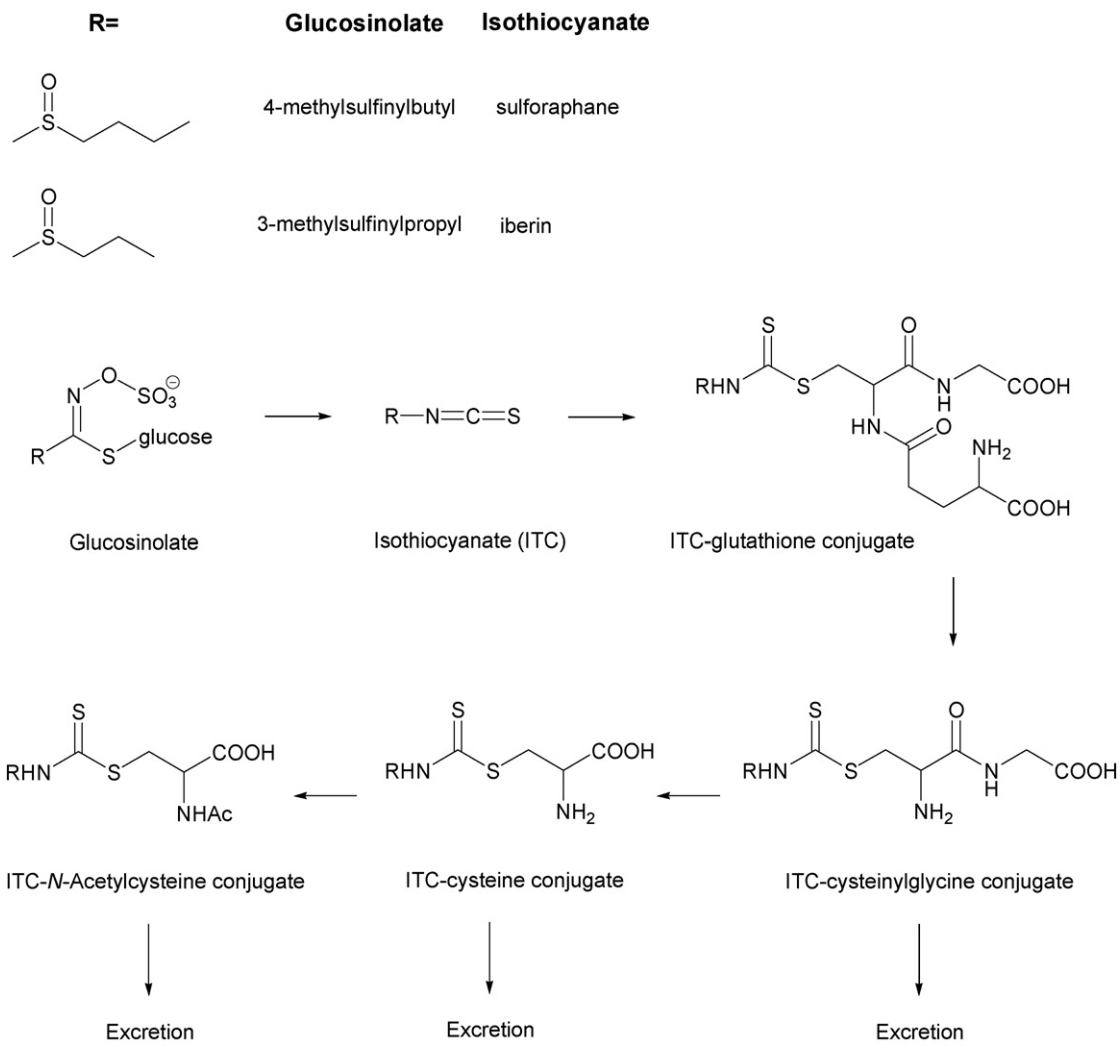

Fig. 1. Chemical structures of the isothiocyanates, sulforaphane and iberin, and their metabolism via the mercapturic acid pathway.

the quantitative analysis of these ITCs and their metabolites in human plasma and urine.

Mennicke et al. [11,12] first measured isothiocyanate conjugates (*N*-acetylcysteine conjugates) in rat urine by thin layer chromatography and subsequently developed the first quantitative method to indirect analyse the metabolites of isothiocyanates using the reaction of released alkyl isothiocyanates with n-butylamine to form corresponding di-substituted thioureas which were then quantified by HPLC. A similar approach was used by Zhang et al. [13] to develop a cyclocondensation reaction of de-conjugated isothiocyanates with 1,2-benzenedithiol to yield 1,3-benzodithiole-2-thione, which can be detected by U.V. spectroscopy or HPLC. While these techniques are useful for assessing overall metabolism, they do not enable the determination of specific isothiocyanate conjugates. The cyclocondensation reaction is not restricted to isothiocyanates but includes dithiocarbamates and related thiocarbonyl compounds such as carbon disulfide, certain substituted thiourea derivatives, and xanthates and hence can potentially give misleading results. More recently, Rose et al. [14] and Vermeulen et al. [15] have reported liquid chromatography–mass spectrometry methods to analyse and quantify isothiocyanates in human urine. While these methods are specific and sensitive they are limited to only one type of isothiocyanate conjugate (*N*-acetylcysteine conjugates). There are no methods reported for the quantitative determination of the mercapturic acid metabolites of sulforaphane and iberin in plasma, with previous analytical methods either being unsuitable or unable to detect the metabolites of sulforaphane and iberin in human plasma [12,14–17].

It is possible that ITC metabolites are present in unquantifiable concentrations in human plasma and three reasons for this can be considered: firstly, the metabolites could be rapidly cleared from plasma and hence never reach measurable concentrations or secondly, the potentially reactive nature of the ITCs could mean that they irreversibly react with plasma proteins via di-sulphide bond formation and hence are not available for detection or thirdly, the conjugates might be unstable in human plasma or during sample processing and hence degrade before analysis. The limited available data on aqueous stability suggests that some of the mercapto-conjugates of phenylethylisothiocyanate and sulforaphane degrade at pH 7.4 at 37 °C [12,17] indicating a potential problem with stability. Another restricting factor in developing quantitative analysis of the mercapto-conjugates has been the lack of commercial availability of authentic stan-

dards, although methods for their synthesis are available in the literature [11,18].

In order to address these issues and optimise methodology for the analysis of these ITCs and their conjugates we have synthesized standards of all the mercapto-conjugates and several potential internal standards, undertaken stability studies in aqueous solution and in human plasma and assessed carefully the optimum analytical approach to achieve meaningful data in human biokinetic studies of dietary sulforaphane and iberin. We present here two sensitive and specific LC–MS/MS assay methods to analyse sulforaphane and iberin and their mercapto-conjugates (glutathione, cysteine, cysteine-glycine, *N*-acetylcysteine) in both human plasma and urine. Data from a human dietary study of broccoli consumption is presented to demonstrate the successful application of the methods.

2. Experimental

2.1. Materials and Methods

Sulforaphane and iberin were obtained from LKT Laboratories, Inc. (Minnesota, USA). Methyl-, ethyl- and butyl-isothiocyanates, cysteine, cysteine-glycine, glutathione, *N*-acetylcysteine were purchased from Aldrich (UK). The method described by Mennicke et al. [11] was used to synthesise benzyl-, ethyl- and butyl- thiocarbamoylcysteine for evaluation as potential internal standards for this study. The synthesis of cysteine, cysteine-glycine, glutathione, *N*-acetylcysteine conjugates of sulforaphane and iberin was by an optimised protocol based on that of Kassahun et al. [18]. The structures of the synthesized materials were confirmed by ^{1}H NMR ^{13}C NMR and LC–MS–MS. All solvents and other chemical used were of HPLC grade and purity was assessed to be 95% or greater in all compounds. Water was obtained from an Elga Maxima water purification system (Elga Ltd., High Wycombe, UK).

2.2. Sample preparation

Urine (1 mL) was filtered (0.2 μm pore size) and a 100 μM aqueous solution of the internal standard (*N*-acetyl-*S*-(*N*-butylthiocarbamoyl)-L-cysteine was added (50 μL). The mixture was diluted to 10 mL with the addition of pre-cooled (4 °C) ammonium acetate buffer (pH 4). The diluted urine (10 μL) was injected directly for analysis. To the frozen plasma sample (0.5 mL), in a 1.5 mL Sure-Lock micro centrifuge tube (Fisher, UK) an aliquot of the internal standard solution (100 μM; 50 μL) was added followed by mixing (30 s), the temperature being kept at 4 °C. Pre-cooled (4 °C) trifluoroacetic acid (50 μL) (TFA) was added to precipitate plasma proteins in the sample followed by brief mixing and refrigerated centrifugation at 11,600 × *g* for 5 min. The resulting supernatant was filtered (0.2 μm pore size) and 10 μL injected for analysis.

2.3. Instrumentation and analytical conditions

2.3.1. LC–MS–MS analysis

A Quattro Ultima tandem mass (Waters Micromass, Manchester, UK) and an Agilent 1100 LC equipped with binary pump, degasser, cooled autosampler and column oven (Agilent Technologies, Waldbronn, Germany) were used for the LC–MS analysis. The HPLC column was a Zorbax SB-Aq 3.5 μm (100 × 2.1 mm). The flow rate was 0.25 mL/min and gradient elution was used: solution A (13 mM ammonium acetate adjusted to pH 4 with 0.1% acetic acid): solution B (acetonitrile + 0.1% acetic acid). The gradient started at 5% solution B increasing over 5 min to 30% B and finally re-equilibrated to 5% B for 6 min. The mobile phase solutions were filtered through 0.45 μm pore size cellulose nitrate or nylon membrane filters using a vacuum filtration apparatus.

The LC eluent flow of 0.25 mL/min was sprayed into the mass spectrometer interface without splitting. Electrospray ionisation in the positive mode (ESI+) was applied. Nitrogen gas flows of 153 and 756 L/h were used for cone gas and desolvation gas flow, respectively. The MS resolution was set to 0.7 amu (full-width at half-maximum peak height, FWHM). The source temperature and solvent desolvation temperature were set at 125 and 350 °C, respectively. Dissociation of the precursor ions was obtained in the collision cell with argon gas. The analytes were detected by tandem MS using multiple reaction monitoring (MRM). The specific cone voltage and collision energy of each analyte were optimised to maximise the ion currents of the selected precursor and product ions using the QuanOptimise facility of the MassLynx (Micromass, Manchester, UK) operating software. The optimisation of the precursor to product ion dissociation was performed by infusion into the MS of separate standard solutions (1 μg/mL) of each analyte. Table 1 summarises the monitored

Table 1
LC–MS/MS parameters for sulforaphane, iberin and their mercapturic acid conjugates

Analyte abbreviation	Name	Retention time (min)	Precursor ion (*m/z*)	Selected product ion (*m/z*)
IB	Iberin	6.8	164.0	105.0
SFN	Sulforaphane	9.1	178.0	114.0
BNAC (IS)	Butyl-ITC-*N*-acetylcysteine	9.3	279.2	122.2
IB-Cys	Iberin-cysteine	2.5	285.3	164.0
SFN-Cys	Sulforaphane-cysteine	3.4	299.2	178.0
IB-Cys-Gly	Iberin-cysteineglycine	2.8	342.2	164.0
SFN-Cys-Gly	Sulforaphane-cysteineglycine	3.9	356.2	179.2
IB-NAC	Iberin-*N*-acetylcysteine	4.4	327.0	164.0
SFN-NAC	Sulforaphane-*N*-acetylcysteine	6.5	341.0	178.0
IB-GSH	Iberin-glutathione	3.2	471.2	179.2
SFN-GSH	Sulforaphane-glutathione	4.6	485.2	179.2

protonated ions and the optimised MS operating parameters of the analytes. The MassLynx software version 4 was used for further data processing.

2.4. Method validation

Blank plasma and urine from five different individuals were extracted and analysed for endogenous substances which could potentially interfere with the determination of the analytes. Intra-day accuracy and precision were evaluated by analysis of replicate spiked blank urine or plasma samples at concentrations of 0.125, 1.0 and 5.0 μM in human plasma and 0.3, 30 and 300 μM in human urine ($n = 6$ at each level) on the same day. To assess the inter-day accuracy and the precision, replicate spiked samples ($n = 6$) were analysed on six different days. The precision was calculated from the relative standard deviation (R.S.D.%) of the replicate analyses. Accuracy was calculated by comparison of expected concentrations with the measured concentration of the spiked samples. A R.S.D.% of 15% was deemed acceptable for both precision and accuracy.

The analyte recovery was calculated by comparing the peak area of the extracted samples to the peak area from the unextracted standard solutions of equivalent concentration prepared in mobile phase. Blank urine and plasma samples were spiked with sulforaphane, iberin and their glutathione, cysteine, cysteine-glycine and *N*-acetylcysteine conjugates at concentrations of 0.3, 30 and 300 μM (urine) and 0.125, 1 and 5 μM (plasma) ($n = 6$ at each concentration).

Calibration standards for each analyte were constructed over the range of 0.025–15 μM in human plasma and 0.01–300 μM in human urine ($n = 6$ at each level) using linear regression analysis (no weighting) from plots of the analyte/internal standard area ratio against concentration. The lower limit of quantitation (LLOQ) was determined as the lowest concentration of replicate analyses for intra-day precision and accuracy ($n = 6$) at which the R.S.D.% was $\leq 20\%$.

2.5. Assessment of stability of sulforaphane and iberin conjugates

The stability of the ITC conjugates (100 μM) was evaluated at pH 12, 7.4, 5 and 2, and at different temperatures to simulate the body temperature (37 °C), room temperature (25 °C) and refrigerator temperature (4 °C). Sodium phosphate (pH 12, 7.4, 2) and ammonium acetate (pH 5) buffers (10 mM) were used. In addition, the stability in human plasma of the ITC conjugates (100 μM) was assessed at temperatures of 37, 25 and 4 °C.

For the measurement of stability of mercapto-conjugates of sulforaphane and iberin in human plasma the LC–MS method described above was used. In assessing the aqueous stability of the conjugates an HPLC–UV method was used involving an Agilent 1090 (Agilent Technologies, Waldbronn, Germany) HPLC system, with an autosampler and UV detector (diode array) set at a wavelength range between 210 and 260 nm was used. A Zorbax SB-Aq 3.5 μm (100 × 4.6 mm) column (Agilent Technologies, Waldbronn, Germany) was used at 40 °C and the mobile phase conditions were as described previously for the LC–MS method except that a flow rate of 1 mL/min was used. The software package Agilent Chem Station for LC 3D (Agilent Technologies, Waldbron, Germany) was used to quantify the amounts of sulforaphane, iberin and their metabolites. GraphPad Prism version 3.0 was used to process the analytical data to calculate the decomposition half-life ($t_{1/2}$) and other related parameters. The stability was expressed as the percentage of the initial (time 0) value using an average of three replicate experiments.

Table 2
Decomposition half-life (h) of sulforaphane metabolites at: (a) 37 °C in buffered aqueous solution; (b) 25 °C in buffered aqueous solution; (c) 4 °C in buffered aqueous solution

Compounds	pH			
	12	7.4	5.0	2.0
(a)				
SFN-NAC	<0.1	10.70	40.70	Stable
SFN-GSH	<0.1	0.30	28.23	Stable
SFN-Cys-Gly	<0.1	0.24	14.22	Stable
SFN-Cys	<0.1	0.16	5.25	Stable
IB-NAC	<0.1	26.5	380.0	Stable
IB-GSH	<0.1	0.29	31.20	Stable
IB-Cys-Gly	<0.1	0.23	13.76	Stable
IB-Cys	<0.1	0.16	5.15	Stable
(b)				
SFN-NAC	0.31	147.29	Stable	Stable
SFN-GSH	<0.1	23.25	163.0	Stable
SFN-Cys-Gly	<0.1	8.07	134.50	Stable
SFN-Cys	<0.1	4.27	79.38	Stable
IB-NAC	0.27	88.37	Stable	Stable
IB-GSH	<0.1	29.21	172.8	Stable
IB-Cys-Gly	<0.1	6.91	129.46	Stable
IB-Cys	<0.1	5.98	90.50	Stable
(c)				
SFN-NAC	<0.1	Stable	Stable	Stable
SFN-GSH	<0.1	40.98	Stable	Stable
SFN-Cys-Gly	<0.1	37.61	Stable	Stable
SFN-Cys	<0.1	28.77	Stable	Stable
IB-NAC	<0.1	Stable	Stable	Stable
IB-GSH	<0.1	79.24	Stable	Stable
IB-Cys-Gly	<0.1	36.46	Stable	Stable
IB-Cys	<0.1	28.51	Stable	Stable

Stable: no detectable degradation over 72 h, $n = 3$ at each time point.

Table 3
Effect of temperature on the stability of sulforaphane metabolites in human plasma

Compounds	Decomposition half-life (h) of SFN and IB metabolites in human plasma		
	Temperature		
	37 °C	24 °C	4 °C
SFN-NAC	2.17	6.16	30.0
SFN-GSH	0.31	1.89	14.0
SFN-Cys-Gly	0.24	0.94	7.61
SFN-Cys	0.16	0.49	2.32
IB-NAC	2.21	4.95	36.7
IB-GSH	0.33	1.59	16.0
IB-Cys-Gly	0.23	0.91	7.14
IB-Cys	0.12	0.52	2.27

$n = 1$ at each time point.

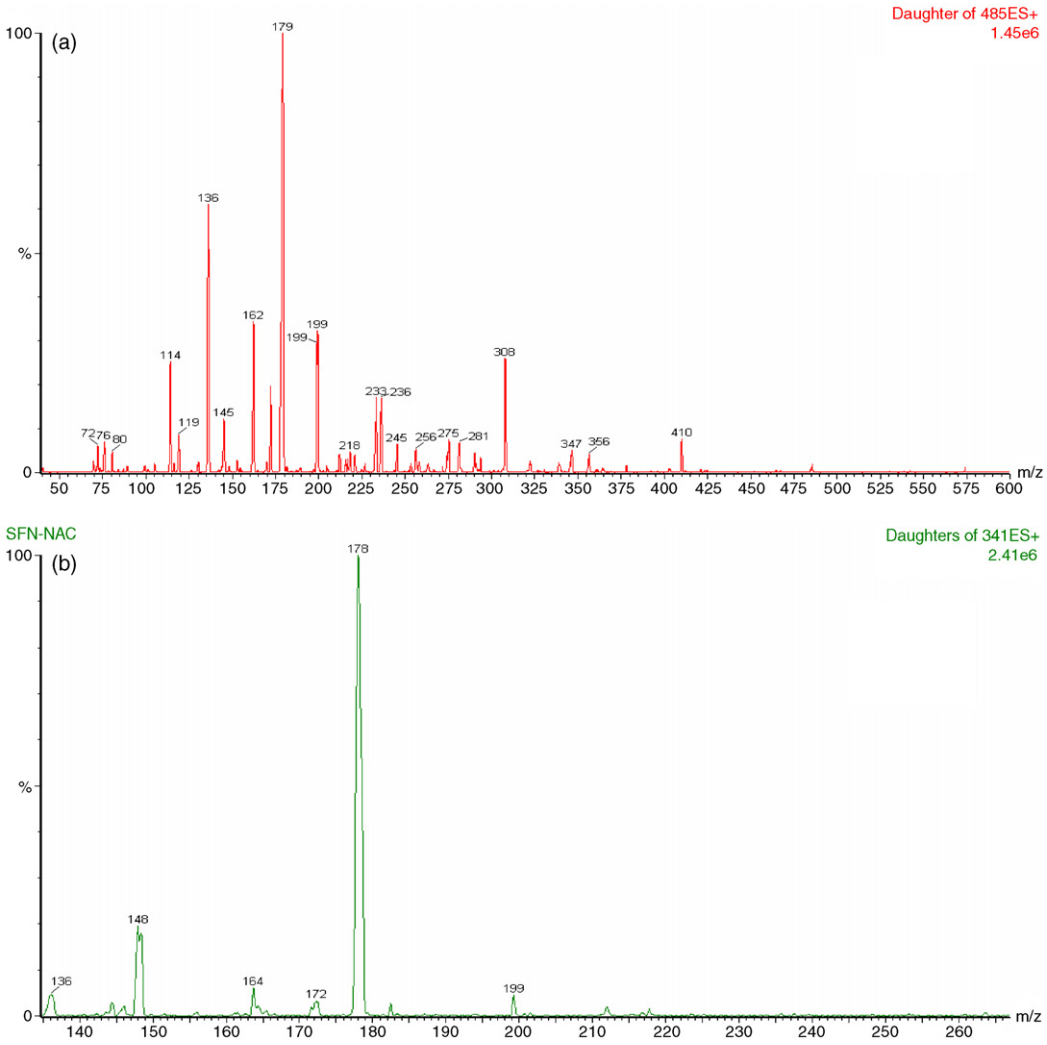

Fig. 2. Examples of different MS–MS dissociation product ion spectra for two sulforaphane metabolites (a) SFN-GSH and (b) SFN-NAC. Spectra of the type (b) were typical for all mercapto conjugates of SFN and IB apart from the SFN-GSG and IB-GSH. Product ion mass spectra were obtained by collision induced dissociation of the protonated molecular $(M+1)^+$, 1 μg/mL reference injected.

Table 4
Validation results of LC–MS/MS analysis of sulforaphane, iberin and their mercapturic acid metabolites in human plasma

Compound	R^2	Slope	Precision ($n=6$) (intra-day) R.S.D.%			Precision ($n=6$) (inter-day) R.S.D.%			Accuracy (R.S.D.%)			Recovery (%)			LOQ (nM)
			L	M	H	L	M	H	L	M	H	L	M	H	
SFN-NAC	0.960	2.20	3.9	2.8	3.4	9.3	7.9	13.1	100	102	104	89	93	92	10
IB-NAC	0.972	1.67	6.9	2.4	2.6	10.0	8.3	9.7	99	101	102	92	94	96	10
SFN	0.999	0.217	3.9	3.6	1.9	3.0	1.4	7.8	100	100	99	85	91	94	15
IB	0.973	0.12	11.4	7.1	3.7	4.6	1.3	9.7	99	100	103	87	90	93	20
SFN-Cys	0.999	0.152	3.3	1.6	4.2	9.9	10.8	7.3	99	100	100	92	95	97	20
IB-Cys	0.999	0.035	2.2	1.2	2.8	9.5	7.3	7.2	99	100	99	82	87	90	20
SFN-GSH	0.994	0.0196	4.2	7.7	6.7	12.8	14.9	13.0	99	99	97	84	89	94	100
IB-GSH	0.998	0.0165	0.9	0.7	6.3	13.1	10.8	14.4	100	99	99	89	92	90	100
SFN-Cys-Gly	0.999	0.0891	4.5	1.5	5.8	7.4	9.8	9.2	100	99	100	92	94	97	20
IB-Cys-Gly	0.981	0.0469	6.7	4.9	6.1	8.6	8.5	6.6	101	100	101	89	92	99	20

Blank plasma was prepared with three different concentrations of all analytes 125 nM (L), 1.0 μM (M), 5.0 μM (H) and measured six times to determine the R.S.D.% of the intra-day precision, accuracy and the recovery. Inter-day R.S.D.% data was obtained on six different. The LOQ was the lowest concentration of each analyte in blank plasma to give ≤20 R.S.D.% ($n=6$).

Table 5
Validation results of LC–MS/MS analysis of sulforaphane, iberin and their mercapturic acid metabolites in human urine

Compound	R^2	Slope	Precision ($n=6$) (Intra-day) R.S.D.%			Precision ($n=6$) (Inter-day) R.S.D.%			Accuracy (R.S.D.%)			Recovery (%)			LOQ (nM)
			L	M	H	L	M	H	L	M	H	L	M	H	
SFN-NAC	0.999	0.250	3.1	2.3	2.3	9.8	5.4	10.2	99	101	94	93	96	96	15
IB-NAC	0.998	0.168	4.4	3.1	2.2	9.2	11.3	9.8	92	96	98	100	92	89	20
SFN	0.989	0.437	1.2	0.6	0.8	4.0	1.7	7.9	101	109	99	95	99	100	20
IB	0.997	0.108	3.4	6.3	0.4	10.0	5.2	3.5	99	100	96	82	89	93	25
SFN-Cys	0.999	0.037	1.6	1.8	0.7	4.6	2.4	1.9	99	99	99	97	85	80	25
IB-cys	0.993	0.031	1.8	2.3	1.4	5.3	3.0	2.4	99	99	109	93	81	83	25
SFN-GSH	0.995	0.0237	1.2	0.1	0.2	3.1	5.3	4.6	99	98	97	94	97	81	150
IB-GSH	0.998	0.0127	1.6	3.0	0.2	2.4	6.1	2.3	99	96	97	93	100	80	150
SFN-Cys-Gly	0.990	0.048	2.0	5.7	7.9	3.0	3.7	1.9	100	104	106	92	86	80	25
IB-Cys-Gly	0.985	0.0123	9.4	9.3	4.4	5.8	5.4	5.1	100	101	108	93	81	83	25

Blank urine was prepared with three different concentrations of all analytes 300 nM (L), 30 μM (M), 300 μM (H) and measured six times to determine the R.S.D.% of the intra-day precision, accuracy and the recovery. Inter-day R.S.D.% data was obtained on six different. The LOQ was the lowest concentration of each analyte in blank plasma to give ≤20 R.S.D.% ($n=6$).

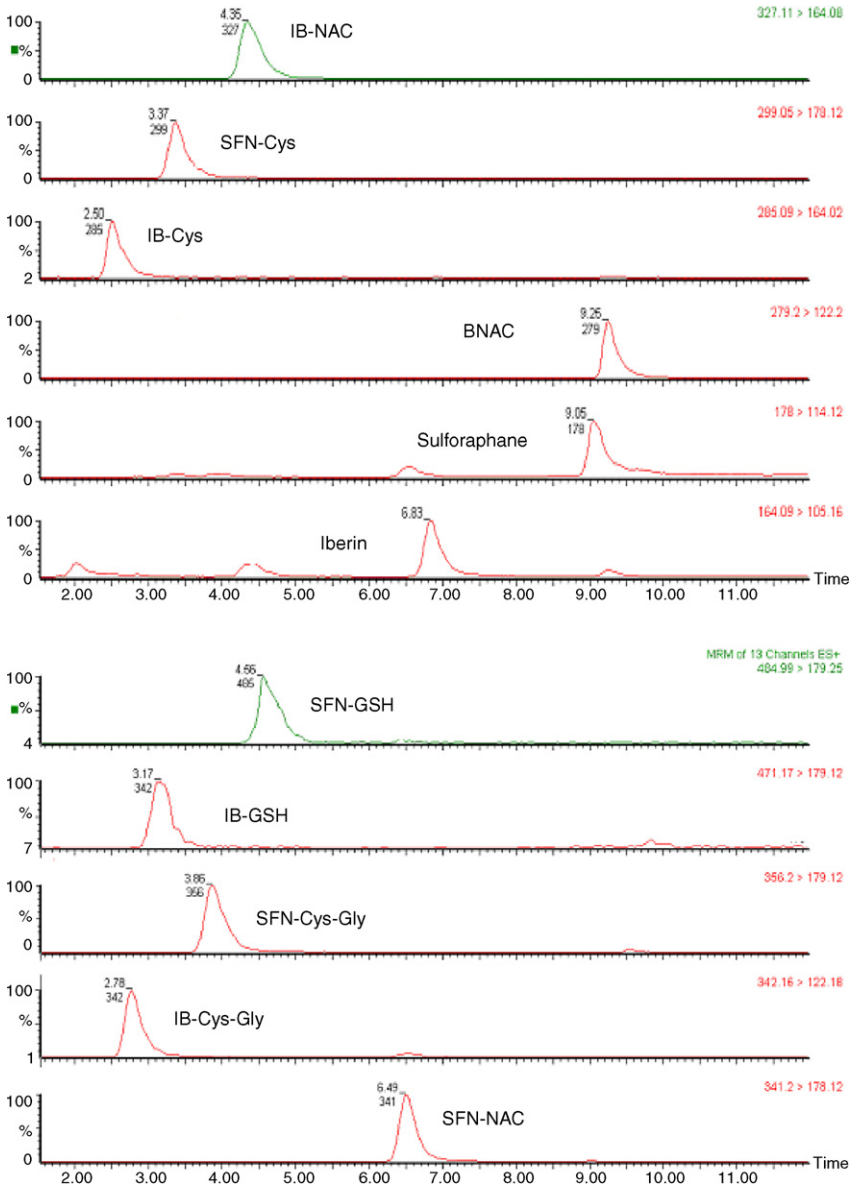

Fig. 3. LC–MS/MS chromatograms of human blank plasma spiked with standards of sulforaphane, iberin and mercapturic acid pathway metabolites (10 μmol/L of each). Mobile phase acetonitrile and 0.01% acetic acid: ammonium acetate adjusted to pH 4 with acetic acid and Zorbax SB-Aq 3.5 μM (100 × 2.1).

2.6. Biological application: biokinetic studies in human volunteers

Sixteen subjects consumed a test meal of broccoli soup (150 mL) containing 100 g of broccoli. Baseline samples of blood and urine were obtained from the subjects after they had avoided foods known to contain glucosinolates, ITCs, spicy foods and alcohol for 24 h prior to the start of the study. Blood samples were collected in tubes that contained lithium heparin at 0, 20, 40, 60, 100 min, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 24 h. Urine was collected between 0–2, 2–4, 4–6, and 6–24 h after consumption. This was part of a larger study which is described in more detail in Gasper et al. [19].

3. Results

3.1. Stability of ITC conjugates in aqueous solution and in human plasma

The pH and temperature values of the aqueous stability study were selected to simulate a range of conditions relevant to both physiological and physical environment that samples might be exposed to. We observed that the *N*-acetylcysteine, glutathione, cysteinylglycine, and cysteine conjugates of sulforaphane and iberin conjugates were rapidly and completely degraded at pH 12 within a minute (Table 2(a)–(c)). All the conjugates showed increasing stability as the pH became more acidic, with maximum stability observed at pH 2. The *N*-acetylcysteine conjugates were most stable followed, in order of stability, by the GSH, Cys-Gly and Cys conjugates for both sulforaphane and iberin. The expected effect of temperature on stability was observed, the actual values of half-life being calculated to assess the potential for any meaningful degradation taking place under physiological and analytical conditions. These results are consistent with previous reports that ITC-mercapto-conjugates are not stable in neutral or mildly alkaline solutions [12,20]. All conjugates in general showed improved stability at pH 5 or lower and at a lower temperature. Kassahun et al. [18] showed similar stability data for SFN-GSH and SFN-Cys, but only over 4 h and also showed that these conjugates underwent thiol exchange in aqueous solution in the presence of excess cysteine. However, any thiol

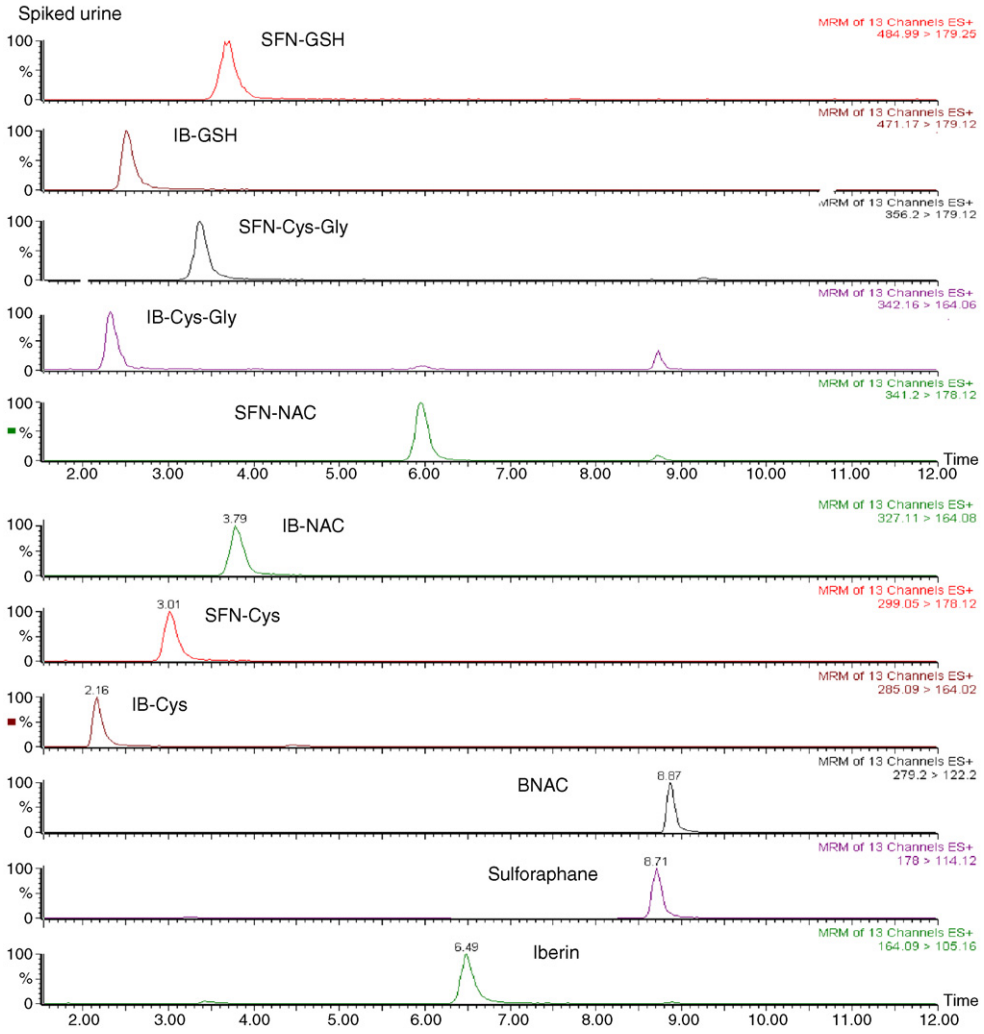

Fig. 4. LC–MS/MS chromatograms of human blank urine spiked with standards of sulforaphane, iberin and mercapturic acid pathway metabolites (10 μmol/L of each). Mobile phase acetonitrile and 0.01% acetic acid: ammonium acetate adjusted to pH 4 with acetic acid and Zorbax SB-Aq 3.5 μM (100 × 2.1).

exchange is unlikely to be significant under in vivo conditions [18].

Table 3 shows the decomposition half lives of sulforaphane and iberin metabolites in human plasma at 37, 25 and 4 °C, respectively, for *N*-acetylcysteine, glutathione, cysteinyl-glycine, and cysteine conjugates. The rapid degradation of the Cys-, CysGly- and GSH- conjugates at 37 °C in plasma suggests that a major reason for the lack of detection of these conjugates may be due to their degradation during sample handing. It is important to note that all these conjugates have a much improved stability at 4 °C, hence rapid cooling of blood samples must be a necessary part of the sample handling protocol.

These stability measurements were used to devise methods to minimise chemical degradation of the conjugates during sample collection and processing. Dilute HCl was pre-added to urine collection vessels to adjust the collected urine to pH 4 or less. Care was taken to ensure that all sample processing was done at pH 4 or less. From these data it was concluded that blood samples should be taken into pre-cooled tubes and that the separation of blood plasma be undertaken in a refrigerated centrifuge (4 °C) prior to storage at −80 °C. In addition, we recommend that any processing of samples should be done under cooled conditions and at acidic pH to prevent unwanted breakdown during sample processing and that, during analysis, samples should be kept in a cooled (4 °C) autosampler. For HPLC analysis, even though

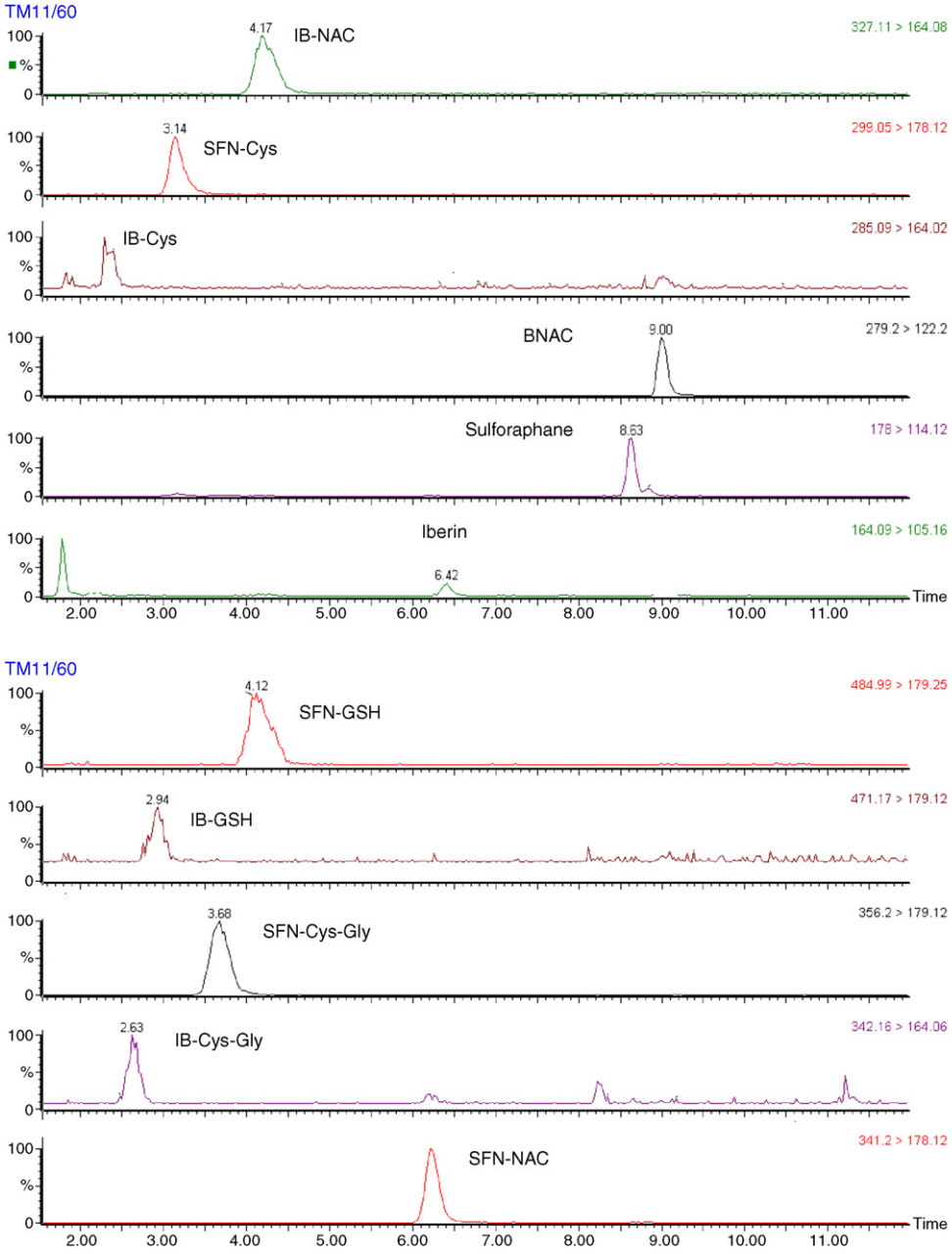

Fig. 5. LC–MS/MS extracted ion chromatogram for sulforaphane, iberin and their metabolites in human plasma at 60 min after broccoli consumption. Mobile phase acetonitrile and 0.01% acetic acid: ammonium acetate adjusted to pH 4 with acetic acid and Zorbax SB-Aq 3.5 μM (100 × 2.1).

the analysis time was relatively short, it was decided to use a mobile phase of around pH 4.0 to minimise any potential for on-column degradation.

3.2. Development of assay methods

The same LC–MS method was used for both plasma and urine and was able to analyse simultaneously sulforaphane, iberin and their mercapto-conjugates in a single analysis without the requirement for repeat injections. Chromatographic resolution was achieved between all the analytes (Table 1), and this was important especially for conjugates containing the same ITC, since there was potential for interference from common fragments derived from breakdown of the ITC-conjugate in the MS source. A standard C18 HPLC column was not able to achieve this separation because the conjugated metabolites were very poorly retained and hence not resolved. A much improved separation was obtained by the use of a Zorbax SB-Aq HPLC column which enabled compete resolution of all the conjugates under high aqueous mobile phase conditions. Little or no interference

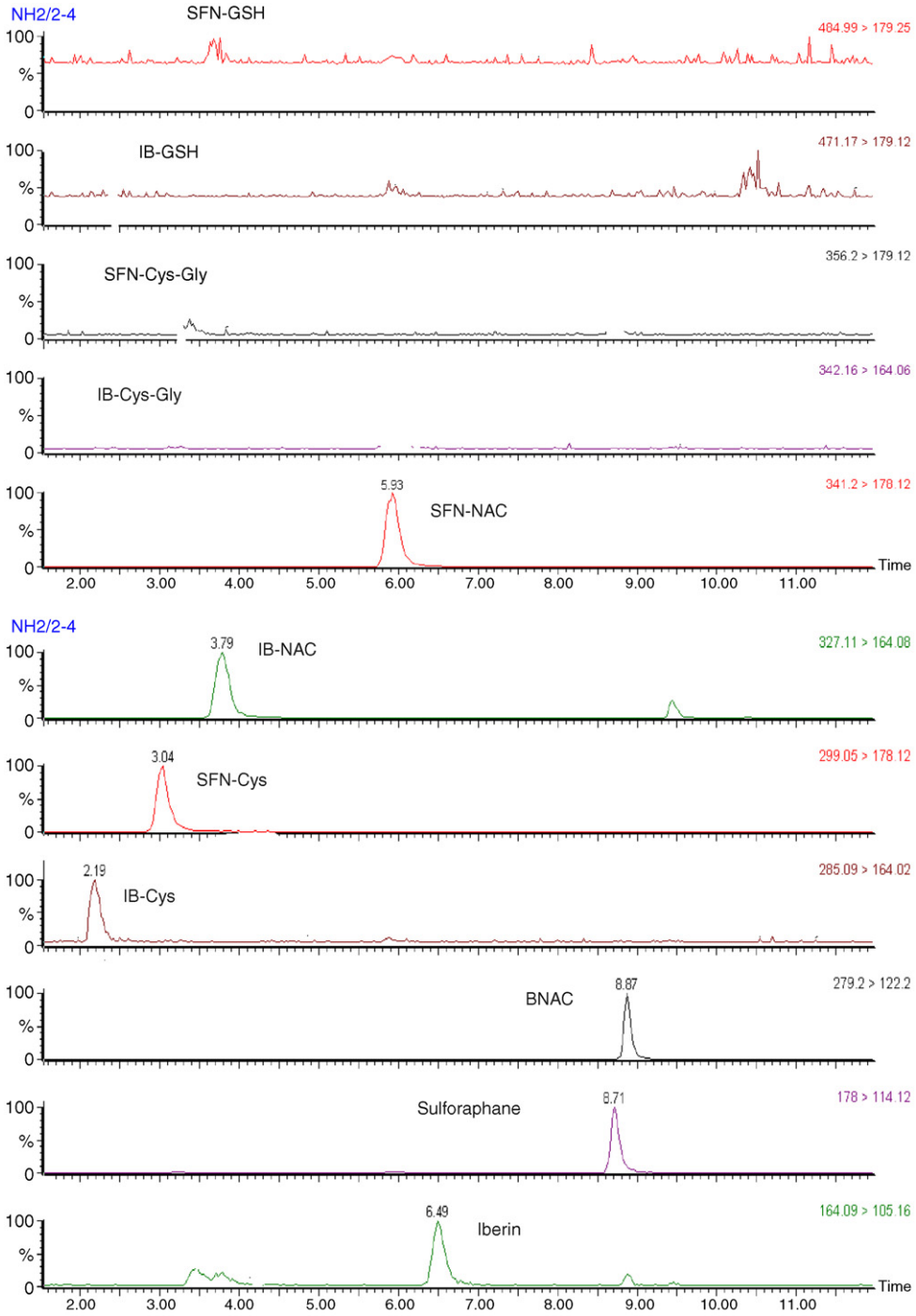

Fig. 6. Sulforaphane, iberin and their metabolites in human urine at 2–4 h after broccoli consumption. Mobile phase acetonitrile and 0.01% acetic acid: ammonium acetate adjusted to pH 4 with acetic acid and Zorbax SB-Aq 3.5 μM (100 × 2.1).

was present in any of the sets of blank human plasma or urine. Minor interfering peaks were observed in some of the MS–MS transitions, but these did not affect the quantitative measurements because they occurred at retention times which did not cause interference with the analytes. Butylthiocarbamoylcysteine was selected as internal standard because its retention time did not cause any interference with the other analytes. For the majority of the ITC conjugates the most abundant fragment ion was the corresponding ITC $[M+1]^+$ ion and this was selected for MRM analysis (Table 1). However, the SFN-GSH and IB-GSH conjugates gave many highly fragmented product ions, and although it was still possible to use the corresponding ITC $[M+1]^+$ ions for MRM purposes (Fig. 2), the sensitivity for the GSH conjugates was reduced by a factor of approximately ten compared with the other conjugates.

Existing methods to extract and prepare isothiocyanates from biological samples are often complicated and time-consuming [12–17]. The aim of this study was to develop a fast and simple extraction procedure suitable for quantitative analysis and high sample throughput. For plasma samples, careful handling was required to avoid degradation of the conjugates during extraction and to reduce losses due to the expected very low concentrations of the ITC conjugates. We evaluated a number of solvent-based extraction methods, but found that these were time-consuming and, in some cases, solvent residues were incompatible with HPLC analysis. We found that protein precipitation with a strong acid to be the method of choice for extraction of plasma samples because of its simplicity and speed, the relatively small dilution involved and its ability to recover high yields of all the polar conjugates as well as the sulforaphane and iberin. Although any strong acid was suitable, we found that trifluoroacetic acid was the most practical because of its volatility and hence compatibility with LC–MS analysis. It was possible to inject the extract following simultaneous removal of the precipitated protein and filtering with no adverse effects on chromatographic separation or peak shape noted due to the presence of trifluoroacetic acid. For the urine samples, where much higher concentrations of the ITC conjugates were known to be present and there was no need to precipitate protein, a simple dilution of filtered urine was found to be the best compromise between speed and sensitivity. The sensitivity of the urine assay method could potentially be improved by using a sample concentration stage, but the trade off would be a much slower sample throughput and we preferred to have faster throughput with adequate sensitivity for the purpose intended.

3.3. Validation of the plasma and urine assay methods

The validation data for the plasma method is shown in Table 4 and for the urine method in Table 5. The results confirm that both methods demonstrate high recoveries of all analytes and are sufficiently linear, sensitive, precise and accurate for application to the measurement of these analytes in human volunteer studies. The intra-run precision for both plasma and urine samples was within an R.S.D.% of 0.9–11.4% and the inter-run was between 1.3 and 13.1%. The values for recovery of sulforaphane, iberin and all ITC conjugates from plasma and urine were greater than 82% at all concentrations (Tables 4 and 5). The linearity of the method was confirmed by analysis of nine-point calibration standards over the range 0.01–300 μM in human urine and 0.025–15 μM in human plasma. The LLOQs for the GSH conjugates in plasma and urine were notably worse that those of the other conjugates, and this was explained by the much reduced abundance of the selected product ion already discussed in an earlier section. However, the LLOQ values for all the metabolites, including the GSH conjugates were considered suitable for profiling biokinetic parameters in humans based on previously available data. The LLOQ values reported here

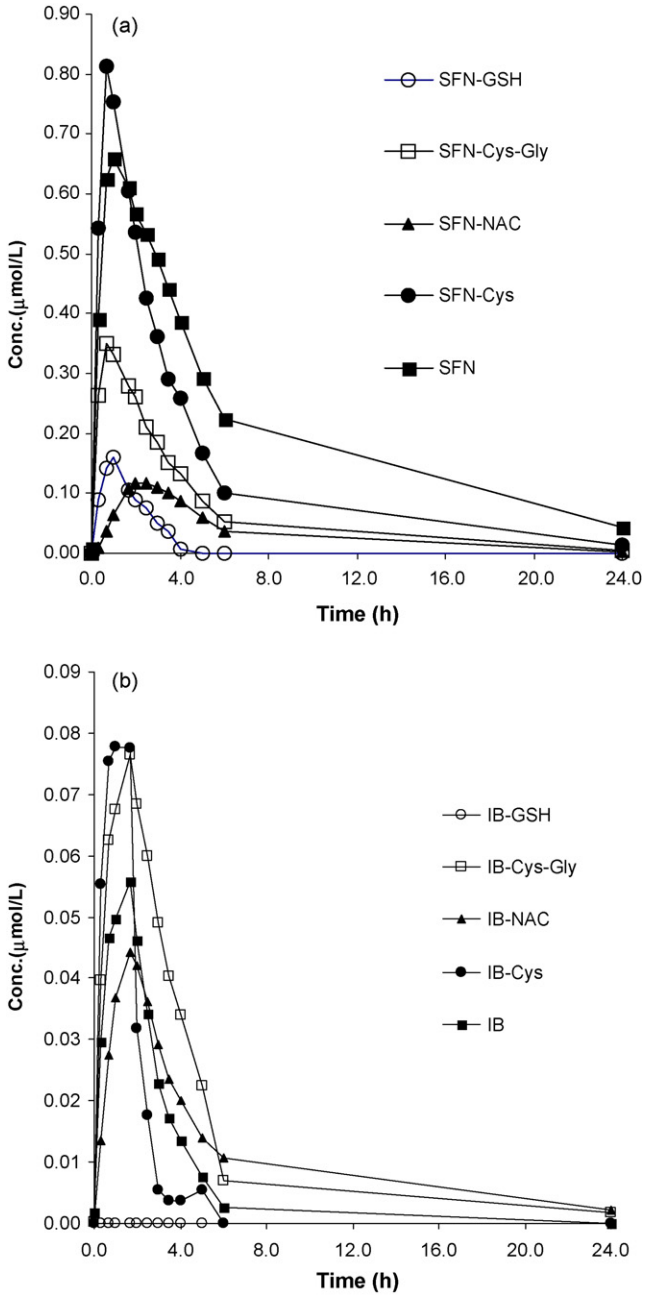

Fig. 7. Plasma concentration vs. time profile for (a) sulforaphane and its mercapturic acid metabolites and (b) iberin and its mercapturic acid metabolites following oral administration of broccoli soup.

are comparable with methods used to investigate phenylethyl isothiocyanate in human plasma and urine by ammonia derivatization and liquid chromatography–tandem mass spectrometry (LLOQ was 7.8 nM) [21], and much improved compared with the published cyclocondensation method (LLOQ was 98 nM) [22]. Figs. 3 and 4 show typical LC–MS/MS chromatograms obtained from the analysis of sulforaphane, iberin and their mercapturic acid conjugates in blank human plasma or urine spiked at a concentration of 10 μM.

3.4. Application of the methods to a human volunteer study

The validated LC–MS–MS methods were successfully applied to analyse timed plasma and urine samples from a human volunteer study in which 100 g of broccoli was consumed. Mass chromatograms obtained from typical plasma and urine samples are shown in Figs. 5 and 6. In plasma it was possible to define complete concentration–time biokinetic profiles for sulforaphane iberin and all the mercapturic acid pathway metabolites, apart from IB-GSH which was not detected in the majority of samples (Fig. 7(a) and (b)). This is the first method to report measurable concentrations of mercapturic acid metabolites of ITCs in human plasma following consumption of broccoli or other dietary sources of ITCs. Our results suggest that the main reason for the previous lack of reporting of these mercapto-ITC metabolites in plasma is due the rapid degradation of these compounds in human plasma. By determining the plasma stability of the conjugates in vitro we were able to design an appropriate extraction method to avoid unwanted breakdown of the ITC conjugates and hence demonstrate their presence in human plasma in vivo. A second reason for the ability of this method to detect the mercapto-conjugates in plasma is the improvement in LLOQ and specificity compared with previous methods as a result of using tandem mass spectrometry detection.

In urine it was possible to measure sulforaphane, iberin and their individual NAC, Cys and CysGly conjugates in all samples, but the GSH conjugates were not detectable. Previously, only the NAC conjugate of SFN had been measured in human urine, but it is clear from the data presented here that, although SFN-NAC is the major metabolite in urine, there are significant quantities of SFN, SFN-Cys and SFN Cys-Gly also present (Fig. 8). The method has proved to be reliable and robust with more than 1000 plasma and urine samples successfully analysed from a human volunteer study investigating the role of broccoli-derived ITCs as anti-cancer agents [19].

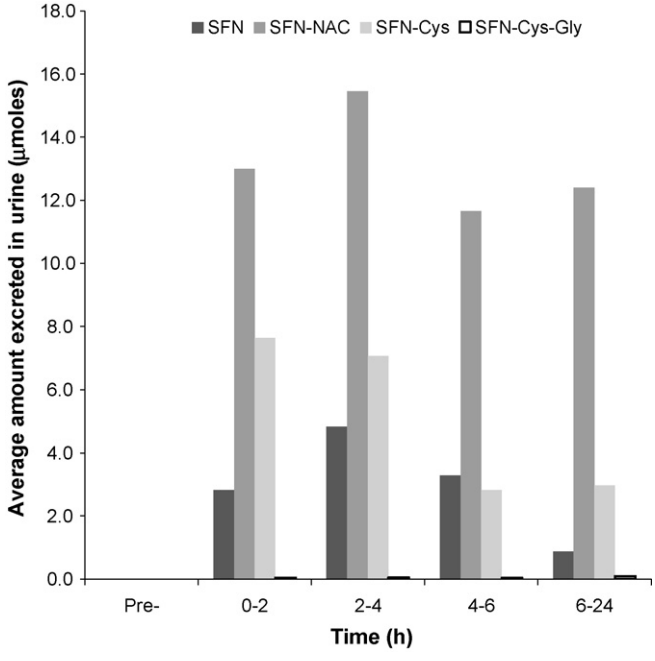

Fig. 8. Urinary excretion of sulforaphane and its mercapturic acid metabolites following oral administration of broccoli soup.

4. Conclusion

We have reported here new methods with significantly improved performance for the analysis of sulforaphane, iberin and their mercapturic acid conjugates in human plasma and urine. The in-depth metabolite profiling now accessible using this methodology will permit more effective and detailed investigations of the effects on human health of dietary sulforaphane and iberin. The methods presented here can also be readily adapted to measure the concentrations of ITCs in dietary material, for example, we have successfully used it to profile ITCs in broccoli soup (data not reported). In addition, the basic analytical approach could be adapted for other types of isothiocyanates and glucosinolate products, since many of these are also known to be metabolised via the mercapturic acid pathway, thereby producing GSH, Cys-Gly, Cys and NAC conjugates.

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