

Quantitative Analysis of Sulforaphane and its Metabolites *in vivo*

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Overview

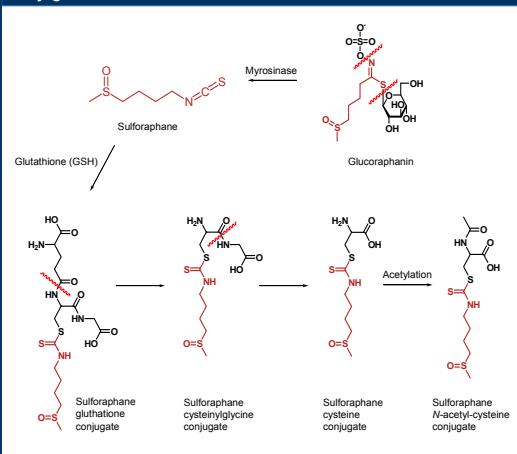
Sulforaphane-glutathione conjugate (SGC) in human serum was investigated by LC-MS/MS. SGC was quantified using multi reaction monitoring (MRM) scanning mode. Selected ion monitoring (SIM), parent and daughter ion scanning techniques were employed to detect and identify other sulforaphane metabolites.

Introduction

Sulforaphane (SFN), a naturally occurring isothiocyanate (ITC), is abundant in cruciferous vegetables and is being investigated as a cancer chemopreventive agent. SFN is a highly potent inducer of enzymes that detoxify carcinogens (phase 2 enzymes). *In vivo*, SFN reacts with a number of compounds, including glutathione (GSH), to produce various conjugated metabolites. Reliable and sensitive analytical methods to determine and quantify SFN and its conjugates in biological matrices are essential for a better understanding of the metabolic fate of SFN and its activity.

SCHEME 1. Sulforaphane glucosinolate metabolic pathway.

Sulforaphane exists in broccoli as glucosinolate. When broccoli is consumed sulforaphane is produced via the action of the myrosinase, an enzyme present in broccoli. After absorption SFN undergoes conjugation with GSH and further transformations¹⁾



Methods

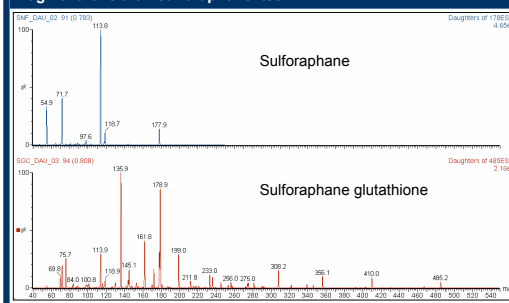
Sample preparation: A male volunteer was fed with a single dose (55g) of 3 day old broccoli sprouts. Blood was collected before and after broccoli consumption. Samples were prepared as described in Figure 1 to produce Pre-B and Post-B samples.

Quantitation assay: Synthetic SGC²⁾ was used for method development and as an external standard to build the calibration curve. For SGC detection and quantitation, analysis was performed in multi-reaction monitoring (MRM) mode, using the transitions 485 to 136, 179 and 308 (see Scheme 2)

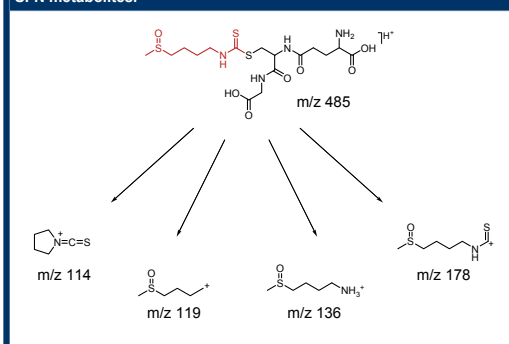
Identification of other SNF metabolites: Analysis in parent ion scanning mode, with 114, 119, 136, 178 m/z ions as precursor ions, were performed for both pre- and post-broccoli serum samples.

Instrumentation: All analyses were carried out by electrospray LC-MS/MS using an Agilent 1100 HPLC and Micromass Quattro Premier triple quadrupole mass spectrometer. QuanLynx software was used for quantitative analysis. HPLC conditions: C18 Targa 40x2.1mm, 3µm; 8 minute gradient run, 0-80%B in 5 minutes; A:0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile; 300µl/min flow rate.

FIGURE 1. MS/MS spectra of SFN and Sulforaphane glutathione. Sulforaphane portion of SGC molecule gives different fragment ions than sulforaphane itself.



SCHEME 2. Sulforaphane glutathione fragmentation. The sulforaphane portion of the molecule produces MS/MS fragment ions of m/z 114, 119, 136 and 178. These ions were used for detection of SFN metabolites.



Results

FIGURE 2. Protein precipitation studies. 100µl of serum adjusted to pH 4 with formic acid (A,C) treated with 300µl of methanol (A,B) or acetonitrile (C,D). Recovered supernatant was dried and reconstituted in 0.1% formic acid prior LC/MS analysis. Quantitation of SGC was performed in MRM mode. Precipitation with MeOH at pH 4 gave the most efficient recovery of SGC.

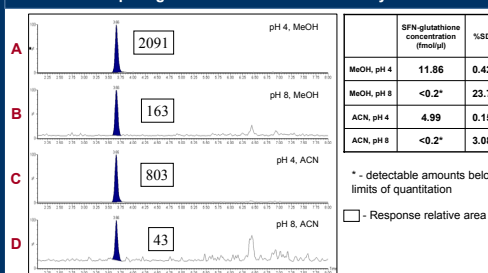


FIGURE 3. SFN metabolite identification. A number of compounds display the characteristic SFN daughter ions. It has been observed that the relative abundance of the various fragment ions is heavily dependent on the MS settings. Absence of the 136 m/z fragment from the 341 and 299 parent ions may be a result of this variability.

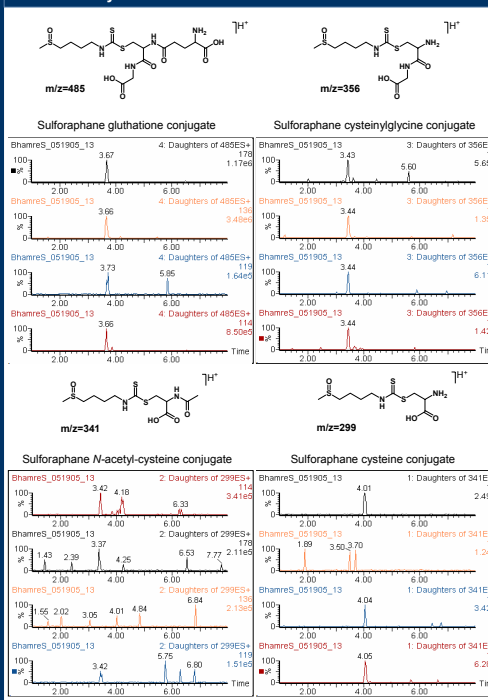


FIGURE 4. SGC quantitation assay. The SGC response was linear over a range of 1-500 fmol, with a correlation coefficient of r=0.998. LOQ was 1 fmol, LOD was 0.1 fmol

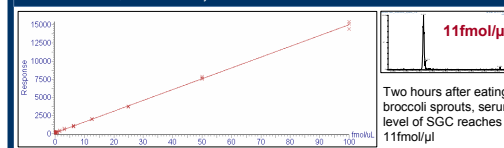
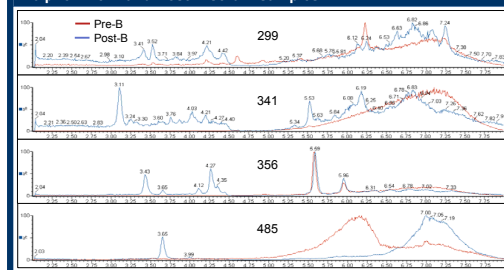


FIGURE 5. Other SFN conjugates. Selected ion monitoring (SIM) enabled the SFN conjugate parent ions (485, 356, 341, 299 m/z) to be extracted from the serum background. Data was collected on 10 µl of Pre-B and Post-B serum samples.



Conclusions

- Detection of sulforaphane glutathione conjugate (SGC) at levels in serum as low as 0.1 fmol/µl is enabled by the very sensitive MRM technique.
- Parent and daughter monitoring allows detection of multiple metabolites, although higher sensitivity is desired.
- Coprecipitation of sulforaphane conjugates with proteins suggests that they are associated with serum proteins. Lowering the pH of the serum prior to the plasma crash prevents coprecipitation.

Future work

- Identification and quantitation of SFN metabolites other than glutathione conjugate in serum, which will enable quantitation of total SFN content.
- Application of the assay to studies of SFN efficacy as a chemopreventative agent in rats.
- Extension of the study to tissue samples, comparing SFN levels in healthy and tumor prostate tissue.

References

- Bruzewitz, G. et al., *Biochem. J.* **1977**, *162*, 99-107
- Kassahun, K. et al., *Chem. Res. Toxicol.* **1997**, *10*, 1228-1233

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