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A derivatization method for the simultaneous detection of glucosinolates and isothiocyanates in biological samples

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ABSTRACT

Various analytical methods have been established to quantify isothiocyanates (ITCs) that derive from glucosinolate hydrolysis. However, to date there is no valid method applicable to pharmacokinetic studies that detects both glucosinolates and ITCs. A specific derivatization procedure was developed for the determination of ITCs based on the formation of a stable *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester derivative, which can be measured by high-performance liquid chromatography with ultraviolet detection after extraction with ethylacetate. The novel method, which is also applicable to the indirect determination of glucosinolates after their hydrolysis by myrosinase, was established for the simultaneous determination of glucoraphanin and sulforaphane. By derivatization, the sensitivity of ITC detection was increased 2.5-fold. Analytical recoveries from urine and plasma were greater than 75% and from feces were approximately 50%. The method showed intra- and interday variations of less than 11 and 13%, respectively. Applicability of the method was demonstrated in mice that received various doses of glucoraphanin or that were fed a glucoraphanin-rich diet. Besides glucoraphanin and sulforaphane, glucoerucin and erucin were detected in urine and feces of mice. The novel method provides an essential tool for the analysis of bioactive glucosinolates and their hydrolysis products and, thus, will contribute to the elucidation of their bioavailability.

Glucosinolates are nitrogen- and sulfur-containing secondary plant metabolites. All glucosinolates have a common core structure comprising a β-D-thioglucose group linked to a sulfonated oxime moiety and a variable side chain derived from amino acids. Depending on the precursor amino acid, glucosinolates are grouped into aliphatic, aromatic, and indolic glucosinolates. Broccoli and other *Brassica* plants contain glucosinolates, some of which have been implicated in the prevention of cancer and other degenerative diseases [1,2]. These effects are attributed to their main hydrolysis products, the isothiocyanates (ITCs),[1] which are formed enzymatically by myrosinase (thioglucosidase, EC 3.2.1.147). Myrosinase is released from plant tissue upon injury to protect the plant from herbivores but is inactivated by thermal treatment. However, biological effects of glucosinolates have also been observed after the intake of cooked *Brassica* vegetables [3–5]. Activation of glucosinolates in the gut is most likely catalyzed by intestinal bacteria [6,7]. To investigate the fate of glucosinolates and their activated products in the organism, there is a need for specific, sensitive, and reliable methods for their quantification.

Various methods for the detection of glucosinolates in plant tissue have been reported previously. These include the indirect determination by photometric measurement of glucose that is released when glucosinolates are hydrolyzed by myrosinase [8], the more specific measurement of desulfoglucosinolates by reversed-phase high-performance liquid chromatography (HPLC) after desulfatation [9,10], or the determination of intact glucosinolates by liquid chromatography coupled to mass spectrometric detection (LC–MS) [11].

The analysis of the main hydrolysis products arising from the glucosinolates, the strong electrophilic ITCs, is difficult because of their volatility and reactivity [12]. Furthermore, ITCs may decompose spontaneously into their corresponding amines [13,14]. Only few methods are available for the detection of glucosinolates and

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[1] *Abbreviations used:* ITC, isothiocyanate; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem MS; N-*t*BOC-Cys-ME, *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester; DAD, diode array detection; UV, ultraviolet.

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ITCs in biological sample material such as plasma and urine. As for the measurement in plant tissue, glucosinolates are usually detected indirectly after hydrolysis with myrosinase in the form of their ITCs. The latter are determined in plasma and urine after cyclocondensation with 1,2-benzenedithiol [15–21]. This method has been proven to be a valuable tool to assess the overall content of glucosinolates and ITCs, although it does not allow separation and identification of individual compounds. Moreover, this derivatization also responds to dithiocarbamates and related thiocarbonyl compounds such as carbon disulfide and substituted thiourea derivatives present in the samples and may lead to an overestimation [22]. It is crucial to distinguish and quantify individual glucosinolates and ITCs in biological samples because the biological activities of glucosinolates and their hydrolysis products differ [23]. For example, glucoraphanin [4-(methylsulfinyl)butyl glucosinolate], the most abundant aliphatic glucosinolate present in broccoli, is activated enzymatically to sulforaphane [4-(methylsulfinyl)butyl ITC], which has attracted much scientific interest due to its beneficial health effects (Fig. 1). Sulforaphane is a potent inducer of phase II detoxification enzymes [24,25], inhibits phase I enzymes [26], and is able to induce cell cycle arrest and apoptosis [27,28]. The measurement of ITCs by gas chromatograph–flame ionization detector (GC–FID) enables the identification of certain ITCs such as sulforaphane [29]. Recently, several methods using LC–MS/MS (tandem MS) have been introduced that provide specific and highly sensitive tools for the measurement of glucoraphanin and sulforaphane [30,31]. However, LC–MS/MS has the disadvantage for a routine analysis of being very expensive, and not every laboratory has access to this technique.

To date, methods to analyze glucosinolates and ITCs have been established only for plasma [20,30,31] and urine [15–21,29] and not for other biological sample material such as fecal samples and gut contents. Nevertheless, as bacterial activation of glucosinolates has been described in the past, measurement of glucosinolates and ITCs in the gut is necessary in order to investigate the fate of glucosinolates in the body.

In the current study, a specific, reliable, and sensitive method for the simultaneous determination of glucosinolates and their corresponding ITCs in biological samples, such as plasma, urine, feces, and gut contents, was established. Owing to its biological relevance, glucoraphanin was selected exemplarily as a representative glucosinolate for this study.

Glucoraphanin was hydrolyzed by myrosinase treatment to sulforaphane and determined indirectly by comparative analysis of samples subjected to enzymatic hydrolysis or not. For the measurement of sulforaphane, which was present in biological samples or formed after myrosinase treatment of glucoraphanin, a derivatization procedure was developed. The applicability of the method was tested by analyzing plasma, urine, feces, and gut contents from mice that received glucoraphanin either as a pure substance or as component of a glucosinolate-enriched diet.

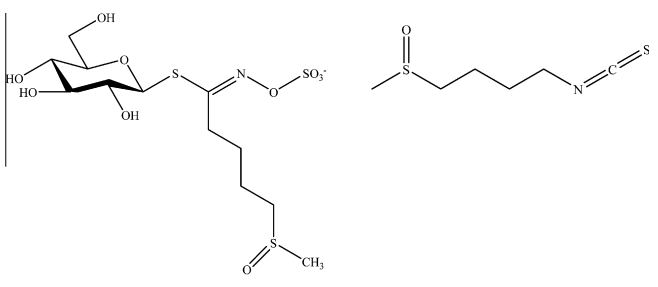

Fig.1. Chemical structures of glucoraphanin and its corresponding ITC sulforaphane.

Materials and methods

Chemicals and enzymes

D,L-Sulforaphane and erucin [4-(methylthio)butyl isothiocyanate] were purchased from Enzo Life Sciences (Lörrach, Germany). Glucoraphanin was obtained from PhytoLab (Vestenbergsgreuth, Germany). Myrosinase, *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester (N-*t*BOC-Cys-ME) and allyl isothiocyanate were purchased from Sigma–Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ethylacetate, acetonitrile, and methanol (HPLC grade) were obtained from Roth (Karlsruhe, Germany). Dimethyl sulfoxide was purchased from Merck (Darmstadt, Germany). All aqueous solutions were prepared with ultrapure water purified with Ultra Clear UV UF (Barsbüttel, Germany). N-*t*Boc-Cys-ME stock solution (1.068 M in methanol) was prepared fresh daily and was further diluted to a 427 mM working solution with 60% (v/v) methanol.

Standards

Standard stock solutions of glucoraphanin and sulforaphane were prepared in ultrapure water to reach final concentrations of 10 mM and stored at −20 °C. Allyl isothiocyanate and erucin were dissolved in dimethyl sulfoxide to obtain a final concentration of 10 mM. For the calibration curve, sulforaphane solutions (0.5–700 μM) were prepared from sulforaphane stock solution in phosphate buffer. Calibration of N-*t*Boc-Cys-ME derivatives of erucin and sulforaphane (0.5–700 μM) were conducted as described below.

Development of derivatization method

Electrophilic ITCs react with the nucleophilic N-*t*Boc-Cys-ME forming a dithiocarbamate ester, which can be separated and detected by HPLC-diode array detection (DAD) [14]. Based on these findings, a reliable method for the determination of these ITC derivatives in biological sample material was established. Conditions for the derivatization were optimized by testing different concentrations of the derivatization agent N-*t*Boc-Cys-ME, different molarities and pH values of the phosphate buffer, and different temperatures and times of reaction as described below.

Calibration and validation of N-tBoc-Cys-ME derivatives

A calibration curve was set up in phosphate buffer (0.1 M, pH 6.7) for sulforaphane and for the N-*t*Boc-Cys-ME derivative by incubating sulforaphane with N-*t*Boc-Cys-ME (21.35 mM) in phosphate buffer under continuous shaking at 300 rpm and 25 °C. After 2 h of incubation, the samples were centrifuged at 20,000*g* and 4 °C for 5 min prior to analysis by HPLC/DAD. The limit of detection, which was defined as a concentration producing a peak height 3 times the baseline noise, was determined to assess the sensitivity of the method.

Applicability of other ITCs to be analyzed with this derivatization method

The applicability of the derivatization method was also tested for other ITCs such as allyl isothiocyanate, which derives from sinigrin, and erucin, which derives from glucoerucin. The derivatization conditions used were those optimized for the derivatization of sulforaphane.