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Direct quantitation of glucoraphanin in dog and rat plasma by LC-MS/MS

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Abstract

A rapid method to quantify levels of the β -thioglycoside N-hydroxyl sulfate, glucoraphanin, in dog and rat plasma to support pre-clinical toxicological and pharmacological studies has been developed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Glucoraphanin was extracted from plasma by protein precipitation with acetonitrile and separated via hydrophilic interaction liquid chromatography (HILIC) using a Luna 5 μ m Silica (2) 100 Å column (50 $\times$ 2.0 mm) at a flow rate of 0.3 mL/min. Solvent A consisted of 200 mM ammonium acetate and formic acid (99:1, v/v) and Solvent B was acetonitrile. Initial conditions (90% Solvent B) were held for 0.01 min after injection, decreased to 40% in 0.5 min and held constant for 2.5 min, returning to initial conditions for 3 min (reequilibration time). Glucoraphanin was detected by MS/MS using a turbo ion spray interface as the ion source operating in negative ion mode. Acquisition was performed in multiple reaction monitoring mode at m/z 435.8 $\rightarrow$ 96.7. The method was validated for the calibration range 10 to 2000 ng/mL. Within- and between-run precision for the low, mid and high QC levels was 8 % R.S.D or less and accuracy ranged from 100 to 113%. The lower limit of quantification was 10 ng/mL; calibration curves encompassed the range of plasma concentrations expected to be found in bioavailability and pharmacokinetics studies with glucoraphanin. The method has successfully been applied to the determination of glucoraphanin in dog and rat plasma and should be extendable to other species as well.

Keywords

Glucoraphanin; Glucosinolate; LC-MS/MS; Rat plasma; Dog plasma

1. Introduction

Glucoraphanin (4-methylsulfinylbutyl glucosinolate) is a β -thioglycoside N-hydroxyl sulfate found in broccoli and other cruciferous vegetables [1]. Through their metabolism to isothiocyanates, naturally occurring glucosinolates have been proposed to confer a broad range of health benefits, including cancer chemopreventive activity. Although the mechanisms

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responsible for the possible cancer chemopreventive activity of glucoraphanin and metabolites have not definitively been determined, these classes of agents may suppress cancer induction through inhibition of p450-mediated activation of chemical carcinogens and/or enhancement of carcinogen detoxification by induction of Phase II enzymes [2,3].

The biotransformation of glucoraphanin to its associated isothiocyanate, sulforaphane, is initiated by hydrolysis catalyzed by the enzyme myrosinase, which is present in the intact tissue of plant material (Fig. 1) [4]. The site and extent of glucoraphanin hydrolysis in the digestive tract are not completely known, although the extent of metabolism appears to depend on the state in which the vegetable is ingested (raw or cooked). More studies using animal models are needed to understand the toxicokinetics and bioavailability of isolated glucoraphanin, as well as its possible mechanisms of action in cancer chemoprevention.

Few methods are available for the determination of glucosinolates. Glucosinolates are generally converted in vivo to their respective isothiocyanates. Thus, in urine, the isothiocyanate metabolites are measured by HPLC with UV detection after cyclocondensation with 1,2-benzenedithiol to form 1,3-benzodithiole-2-thione [5,6]. Similarly, glucosinolates in plant extracts have been determined after hydrolysis with myrosinase and conversion of the resulting isothiocyanates to 1,3-benzodithiole-2-thione [7]. Alternatively, the resulting isothiocyanate can be measured by GC with FID detection [4,8]. Both methods rely on the conversion of glucosinolates to their resulting isothiocyanates, and thus are not specific for the determination of glucoraphanin in the presence of its hydrolysis product sulforaphane. Nevertheless, the indirect measurement of glucosinolates after conversion to isothiocyanates and other dithiocarbamate metabolites present in urine seems to be the preferred approach referred to in recent publications exploring the metabolism, excretion and chemoprevention effects of the compounds in humans [9,10,11].

Direct analysis of glucosinolates in vegetable extracts has been performed using hydrophilic interaction liquid chromatography (HILIC) and ion-pair chromatography with UV detection [12]. Neither of the above methods are applicable to the direct analysis of glucoraphanin in plasma samples. Recently, an LC-MS/MS method for the analysis of glucosinolates, isothiocyanates and amine degradation products in vegetable extracts and human plasma using reversed phase HPLC has been reported [1]. However, in five human subjects, glucosinolates were reported to be below the limit of detection. Quantitation was performed using the standards addition method. No additional information related to the quantitation of glucosinolates in plasma was provided in this report. Similar methods using reversed phase HPLC and tandem mass spectrometry have also been used for the determination of intact and desulfated glucosinolates in plant extracts [13,14]; however, these methods lack the chromatographic performance required for plasma samples.

Two novel approaches were recently published for the determination of total glucosinolates in plant extracts using a flow amperometric enzymatic method [15] and solid phase extraction and micellar electrokinetic capillary chromatography [16]. The applicability of these methods to plasma samples has not been demonstrated.

Initial attempts in our laboratory to use reversed phase chromatography on a C18 column for the analysis of glucoraphanin in plasma samples proved unsuccessful due to the poor retention of the analyte. In this work, we present a validated analytical method for the determination of glucoraphanin in plasma samples using HILIC on a silica gel column with tandem mass spectrometry detection. The method allows for the direct and rapid analysis of glucoraphanin from plasma by LC-MS/MS following precipitation with acetonitrile. Quantitation is performed using the external standard method. The validated method was applied to the

determination of glucoraphanin in dog and rat plasma samples from pre-clinical toxicological and pharmacological studies.

2. Experimental

2.1. Chemicals and reagents

Glucoraphanin sodium salt (purity 99.1%) was obtained from the Chemopreventive Agent repository maintained by the Chemopreventive Agent Development Research Group, Division of Cancer Prevention, National Cancer Institute (Bethesda, MD). Acetonitrile, ammonium acetate and formic acid (all HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). HPLC grade water was generated using a PURELAB Ultra system from ELGA (Lowell, MA) followed by filtration with a Millipore (Billerica, MA) system using a 0.25 μm filter. Blank plasma was obtained from otherwise untreated animals and frozen at -20°C until analysis.

2.2. Animals

Three (3) male and 3 female non-naïve beagle dogs (approximately two and a half years of age; Covance Inc., Cumberland, VA) and twenty-five (25) male and 25 female CD rats [CrI:CD (SD) IGS BR] (received at approximately six weeks of age; Charles River Laboratories, Portage, MI) were used in this study. Prior to experimental initiation the attending veterinarian certified that the animals were healthy and free from disease and parasites.

Dogs were housed individually in pens and rats were housed individually in stainless steel cages. The animals were housed in accordance with standards set forth in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and by the U.S. Department of Agriculture through the Animal Welfare Act (7 USC 2131, 1985) and Animal Welfare Standards incorporated in Title 9, Part 3 of the Code of Federal Regulations, 1991.

Animal rooms were held within a temperature range of approximately $18\text{--}29^{\circ}\text{C}$ and a humidity range of approximately 30–70%. Fluorescent lighting in the animal rooms was provided for 12 hours followed by 12 hours of darkness.

Certified commercial dog or rodent diet was provided once daily (dogs) or ad libitum (rats). City of Chicago municipal water was available ad libitum by automatic watering systems in all pens and cages.

2.3. Preparation of stock solutions and standards

Stock solution of glucoraphanin (1 mg/mL) was prepared by dissolving glucoraphanin in acetonitrile/water (50:50, v/v). Stock solution was stored at -20°C when not in use. Working standards were prepared by further diluting the stock solution in acetonitrile/water (50:50, v/v) to prepare concentrations ranging from 100 to 20000 ng/mL. Calibrators, ranging from 10 to 2000 ng/mL, were prepared by adding 10 μl of the working standards to 100 μl of plasma on each day of analysis.

2.4. Sample preparation

Plasma samples were thawed at room temperature before processing. In a microcentrifuge tube, 1 mL of acetonitrile was added to 100 μl of plasma, to which 10 μl of acetonitrile/water, [50:50, v/v; or a standard or quality control (QC) solution] was also added. The samples were vortex mixed for 1 min. and centrifuged in a Sorvall RC 5C Super Speed centrifuge (Thermo Fisher Scientific, Waltham, MA) at the highest setting for 5 min. The supernatant layer was transferred to a vial for injection into the LC-MS/MS.

2.5. Instrumentation

Samples were analyzed on an API 3000 LC-MS/MS system (Applied Biosystems/MDS Sciex, Foster City, CA) equipped with an Agilent 1100 HPLC (Agilent Technologies, Wilmington, DE). Analyst™ 1.3.2 was used to control the HPLC and mass spectrometer and to capture the mass spectrometer data, perform linear regression analysis and calculate sample concentrations. Separation of glucoraphanin from plasma components was achieved using HILIC with a Luna 5 µm Silica (2) 100 Å column 50 × 2.0 mm (Phenomenex, Torrance, CA). The column temperature was maintained at 25 °C, and a flow rate of 0.3 ml/min. was used. The mobile phase consisted of Solvent A: 200 mM ammonium acetate and formic acid (99:1, v/v) and Solvent B : acetonitrile. The mobile phase gradient was as follows: after injection, initial conditions with Solvent B at 90% were held for 0.1 min., decreased to 40% in 0.5 min. and held constant for 2.5 min., returning to initial conditions for another 3 min. of reequilibration time. Retention time of glucoraphanin was approximately 2.9 min. Total run time was 6 min. A turbo ion spray interface was used as the ion source operating in negative ion mode. Acquisition was performed in multiple reaction monitoring mode using m/z 435.80 (deprotonated molecule, $[M-H]^-$) → 96.70 ($[SO_3H]^-$) at low resolution. Ion spray voltage was −4200 V, ion spray temperature was 500 °C, and dwell time was 300 ms. The collision gas was nitrogen and the collision energy was set at −40 V.

2.6. Method validation

Method validation in dog plasma was performed following the FDA's *Guidance for Industry: Bioanalytical Method Validation* [17] after a full validation in rat plasma. The following factors were used to assess assay performance: selectivity, linearity, precision, accuracy, recovery and stability. The selectivity of the method was assessed by analyzing extract from six individual animals for the presence of analytical interferences and comparing the results to those obtained from spiking the blank plasma sources with glucoraphanin at the lower limit of quantitation (LLOQ; 10 ng/mL). Linearity was assessed using the external standard method and up to eight calibrators with analyte concentrations in the 10 to 2000 ng/mL range. The curves were built from peak areas using least-squares linear regression with $(1/x^2)$ weighting factor. The weighting factor was chosen based on goodness-of-fit criteria including coefficient of determination (r^2), the back-calculated concentration of individual calibrators, and minimizing intercept value. Precision and accuracy of the method were determined from three validation runs with QC samples ($n = 6, 3, 3$ for runs 1, 2 and 3, respectively) prepared at the LLOQ (10 ng/mL), low (25.0 ng/mL), mid (800 ng/mL), and high (1600 ng/mL) concentrations. Within-run precision and accuracy was assessed from the results from a single day, while between-run precision and accuracy were determined from the results from the three validation runs. Extraction recovery of glucoraphanin was determined by comparison of peak area results of the QC samples to peak area results of extracted blank plasma spiked post extraction with glucoraphanin at the same levels. Bench-top stability was determined by analyzing the low and high level QC samples for glucoraphanin concentration after 24 hours of storage at ambient temperature and comparing the results to those obtained from freshly thawed samples. Freeze-thaw stability of the low and high level QC samples was determined over three freeze-thaw cycles. Stability was also determined for low and high level QC samples that were extracted and stored in the autosampler; samples were analyzed and the concentrations determined after approximately one day of storage. To evaluate the impact of dilution on the analyses, six samples were prepared at 8000 ng/mL and diluted 5-fold with blank plasma prior to analysis.

2.7. Application to pre-clinical toxicological study

Glucoraphanin was administered orally by capsule to three male and three female non-naïve beagle dogs once daily for three consecutive days at a dose level of 200 mg/kg/day of body

weight. Plasma samples were obtained from each dog pre-dose and approximately 60 minutes following dosing on the last day (Day 3) of the three-day dosing period.

To evaluate the toxicity of glucoraphanin following oral administration (gavage) to rats for fourteen days, five groups of rats (five males and 5 females per group) were dosed at 0 (control), 10, 50, 100, or 500 mg/kg of body weight daily. Dose formulations were prepared using ASTM Type 1 water as the vehicle. Glucoraphanin was measured in plasma samples collected approximately 60 min. after dosing on Day 13 of the study.

The dog and rat plasma samples were analyzed for levels of glucoraphanin using the analytical method presented here.

3. Method validation results

3.1. Specificity

No significant peaks interfering with the quantitation of glucoraphanin were detected in the chromatograms of blank plasma. Fig. 2A, 2B and 2C show representative chromatograms of blank dog plasma extract, the lower limit of quantification (LLOQ, 10 ng/mL), and a 200 ng/mL calibrator in dog plasma, respectively. Fig. 2D, 2E and 2F show representative chromatograms of blank rat plasma extract, the LLOQ (10 ng/mL), and a 200 ng/mL calibrator in rat plasma, respectively.

3.2. Calibration curve

Calibration curves for glucoraphanin were linear from 10 to 2000 ng/mL. The r^2 values were greater than 0.995. The back-calculated concentration of individual calibrators used to determine the calibration curve ranged from 89 to 112% of the true value. Between-run precision for the back-calculated calibrator concentrations ranged from 1.3 to 4.4% relative standard deviation (R.S.D). Calibrators that fell outside the range of 85 to 115% were not used to calculate the standard curve. Results of the calibration curves are presented in Table 2.

3.3. Precision and accuracy

For the low QC level, within-run precision ranged from 2.7 to 8.3% R.S.D and accuracy ranged from 101 to 107%. Within-run precision for the mid QC level ranged from 1.2 to 5.0% R.S.D and accuracy ranged from 100 to 113%, and within-run precision for the high QC level ranged from 0.7 to 1.8% R.S.D and accuracy ranged from 103 to 108%. Between-run precision and accuracy for the low QC level were 5.4% R.S.D and 103%, respectively. For the mid QC level, between-run precision was 6.0% R.S.D and accuracy was 104%, and for the high QC level between-run precision was 2.6% R.S.D and accuracy was 104%. Precision and accuracy results are presented in Table 3.

3.4. Recovery

Average recovery of glucoraphanin from dog plasma was 58, 53, and 52% for the low (25 ng/mL), mid (800 ng/mL), and high (1600 ng/mL) level QC samples, respectively. Although recovery from dog plasma was relatively low, it was consistent over the range of concentrations investigated. The results of recovery experiments are presented in Table 1.

3.5. Lower limit of quantification (LLOQ)

Precision from the replicate analyses of plasma samples spiked at the LLOQ was 12, 6.2, and 12%, and accuracy was 94, 102, and 96% on the three days, respectively. Between-run precision and accuracy were 10% R.S.D. and 97%, respectively. Results for the LLOQ determination are included in Table 3.

3.6. Bench-top stability

For the low level QC, the change after 24 hours of storage was -6.6% compared to freshly thawed samples, and for the high level QC the change was -3.6%. Results of bench-top stability are presented in Table 4.

3.7. Freeze-thaw stability

The change after three freeze-thaw cycles for the low QC samples was 2.2% and the change for the high QC samples was -6.1% when compared to samples that had been frozen and thawed only once. Freeze-thaw stability results are presented in Table 4.

3.8. Autosampler stability

The change after one day of storage for the low QC samples was -12% and the change for the high QC samples was -8.3% when compared to samples analyzed immediately after extraction. Autosampler stability results are presented in Table 4.

3.9. Sample dilution

For the samples diluted 5-fold above the curve range with blank plasma, accuracy was 88% of the target, with a precision of 3.5%.

4. Discussion

4.1. Method development

Initial attempts in our laboratory to use reversed phase chromatography on a C18 column for the analysis of glucoraphanin in plasma samples proved unsuccessful due to the poor retention of the analyte. The HILIC method gave peaks of sufficient sensitivity in a reasonable retention time.

4.2. Assay performance

We have developed a simple, rapid LC-MS/MS assay for the determination of glucoraphanin in dog plasma samples collected in toxicology and pharmacology studies. To the best of our knowledge, this constitutes the first report for the analysis of intact glucoraphanin in plasma. The method is linear between 10 ng/mL and 2000 ng/mL, a concentration range that encompasses the plasma levels that may be expected to be seen in toxicology, bioavailability, and pharmacokinetics studies with glucoraphanin. We have applied this method to the analysis of glucoraphanin in dog plasma after oral dosing at 200 mg/kg/day of body weight for three days. Glucoraphanin levels in dog plasma approximately 60 minutes following dosing on the third day were in the 2900 to 15000 ng/mL range.

We have also applied this method to the analysis of glucoraphanin in plasma samples from a 14-day oral range-finding toxicity study of glucoraphanin in rats. After dosing 5 groups of rats (5 animals per group per sex) at 0 (control), 10, 50, 100, or 500 mg/kg of body weight daily, glucoraphanin was measured in plasma samples collected approximately 60 min. after dosing on Day 13 of the study. Glucoraphanin was not detected in the plasma of the control group animals. All animals receiving glucoraphanin had measurable levels of glucoraphanin in plasma. The mean measured concentrations for groups dosed at 10, 50, 100, and 500 mg/kg/day were 49.9 ng/mL (range, 13.2 to 83.4 ng/mL); 198 ng/mL (range, 111 to 300 ng/mL); 416 ng/mL (range, 254 to 577 ng/mL); and 1630 ng/mL (range, 1110 to 2470 ng/mL), respectively.

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Fig. 1.
Enzymatic conversion of glucoraphanin to sulforaphane.

Fig. 2.

Fig. 2A. Blank dog plasma.

Fig. 2B. Calibrator LLOQ (10 ng/mL) in dog plasma.

Fig. 2C. Calibrator at 200 ng/mL in dog plasma.

Fig. 2D. Blank rat plasma.

Fig. 2E. Calibrator LLOQ (10 ng/mL) in rat plasma.

Fig. 2F. Calibrator at 200 ng/mL in rat plasma.

Table 1**Extraction recovery results**

Recovery of glucoraphanin from dog plasma was determined by comparison of peak area of QC samples to peak area of extracted blank plasma spiked post extraction with glucoraphanin.

Nominal Glucoraphanin Concentration (ng/mL)		Extracted Sample Peak Area ^a	Reference Sample Peak Area ^a	Glucoraphanin Recovery from Dog Plasma (%)
25.0	Mean	2.87E+03	4.95E+03	58
	SD	5.4E+01	1.4E+02	
	%R.S.D	1.9	2.9	
800	Mean	6.59E+04	1.24E+05	53
	SD	7.9E+02	1.3E+03	
	%R.S.D	1.2	1.1	
1600	Mean	1.35E+05	2.59E+05	52
	SD	2.6E+03	2.8E+03	
	%R.S.D	1.9	1.1	

^aThe number of sample replicates (n) = 6 for all the recovery experiments.

Calibration curves

Table 2

Calibration curves for glucoraphanin were calculated from peak areas using a weighted ($1/x^2$) linear regression. The external standard method was used to calculate the standard curves.

Calibrator Glucoraphanin Concentration	Calibrator Accuracy (%)				Accuracy (%)	
	Run 1	Run 2	Run 3	Mean	SD	%R.S.D
ng/mL						
10.0	107	103	104	10.5	0.21	2.0
20.0	87	91	89	17.8	0.38	2.1
50.0	102	111	107	53.3	2.4	4.4
100	92	93	98	94.1	3.1	3.3
200	98	105	105	205	7.9	3.8
500	101	100	103	506	6.6	1.3
1000	112	-- ^a	-- ^a	1122	-	-
2000	101	98	95	1958	70	3.6
y-intercept	814	1650	1990	1485		
Slope	81.6	98.6	106	95.4	13	13
r ²	0.9958	0.9966	0.9971	0.9965		

^aMeasurement outside the acceptance range; not used in the linear regression analysis of the curve

Table 3
Within-run and between-run precision and accuracy for QC samples

Within-run and between-run precision and accuracy were determined at three concentrations (blank dog plasma). The lower limit of quantification (LLOQ) was determined at the lowest calibrator concentration.

Nominal Glucoraphanin Concentration (ng/mL)	Measured Glucoraphanin Concentration (Blank Dog Plasma) ^a (ng/mL)			
	Run 1	Run 2	Run 3	Between-Run
10 (LLOQ)	Mean	9.39	10.2	9.60
	SD	1.1	0.63	1.1
	%R.S.D	12	6.2	12
	Accuracy (%)	94	102	96
25.0 (Low QC)	Mean	25.1	25.6	26.7
	SD	0.67	2.1	1.6
	%R.S.D	2.7	8.3	5.9
	Accuracy (%)	101	102	107
800 (Mid QC)	Mean	798	905	831
	SD	9.7	13	42
	%R.S.D	1.2	1.5	5.0
	Accuracy (%)	100	113	104
1600 (High QC)	Mean	1643	1728	1644
	SD	30	19	12
	%R.S.D	1.8	1.1	0.70
	Accuracy (%)	103	108	103

^aFor the LLOQ determination, the number of samples (n) = 5, 3, 3 and 11 for Run 1, Run 2, Run 3, and Between-Run, respectively; for the QC samples, n = 6, 3, 3 and 12, respectively.

Table 4**Stability assessment**

Bench-top, freeze-thaw and autosampler stability were determined for low and high QC samples (blank dog plasma).

Nominal Glucoraphanin Concentration (ng/mL)	Mean Measured Glucoraphanin Concentration (Blank Dog Plasma) ^a (ng/mL)			
	Bench-Top Stability			
	24 Hours at Room Temperature	%R.S.D	Reference Samples ^b	% Change
25.0	23.9	2.1	25.6	-6.6
1600	1666	1.5	1728	-3.6
	Freeze-Thaw Stability			
	3 Freeze-Thaw Cycles	%R.S.D	Reference Samples ^b	% Change
25.0	26.1	4.1	25.6	2.2
1600	1622	3.8	1728	-6.1
	Autosampler Stability			
	24 Hours After Extraction	%R.S.D	Reference Samples ^b	% Change
25.0	22.5	7.6	25.6	-12
1600	1584	2.7	1728	-8.3

^aThe number of sample replicates (n) = 3 for all the stability experiments.

^bThe reference samples (n=3) were spiked, extracted and analyzed the same day.