Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 812–816

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Method development for β-glucogallin and gallic acid analysis: Application to urinary pharmacokinetic studies

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ARTICLE INFO

Article history:
Received 6 July 2010
Received in revised form 1 November 2010
Accepted 3 November 2010
Available online 11 November 2010

Keywords:
β-Glucogallin
Gallic acid
Pharmacokinetics
HPLC/ESI/MS

ABSTRACT

A reversed phase high-pressure liquid chromatographic electrospray tandem mass spectrometry method was developed for the simultaneous detection and analysis of β-glucogallin and gallic acid. This method used a C_{18} column with ultraviolet detection at 285 nm, ionization in the negative ion mode for β-glucogallin and gallic acid, and in the positive ion mode for the internal standard 3-morpholin-4-ylpropane-1-sulfonic acid. Mobile phase consisted of a mixture of water, methanol, and formic acid at a flow rate of 0.7 mL/min. This method was validated over a concentration range of 1–100 μg/mL in rat urine.

1. Introduction

There has been an increased interest in both the study and consumption of natural products as evidenced by the increase in nutraceutical sales and the practice of alternative medicine worldwide [1]. Use of these products is substantial through health and beauty, dietary supplement, performance enhancement, food and beverage, to overall health and well being products.

Secondary plant metabolites such as tannins are well known and typically have important roles in plant–plant and plant–animal interaction roles, more specifically in adaptation and aesthetics [2]. Hydrolyzable tannins as opposed to condensed tannins, are a subsection of plant tannins that can be described as esters of gallic acid [3]. β-Glucogallin is also referred to as [(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] 3,4,5-trihydroxybenzoate, 1-galloylglucose, 1-galloyl-β-glucose, 1-galloyl-glucose, and 1-O-galloyl-β-D-glucose, while gallic acid is identified as gallate and 3,4,5-trihydroxybenzoic acid. β-Glucogallin is the glycosylated form of gallic acid, involving UDP-glucose [4]. β-Glucogallin has been identified as the initiating reactant molecule for gallotannins, it is an acyl acceptor and donor, and is an energy-rich polyphenol that may play an integral role in translocation reactions as determined by enzymology studies [3–6]. β-Glucogallin and/or gallic acid have been detected and/or isolated from a variety of botanicals [5–10].

In this study we focus on the development of a novel, accurate, sensitive, reproducible, method to simultaneously quantify β-glucogallin and gallic acid. Ultimately the development of this method will allow us to further study the disposition of both β-glucogallin and gallic acid in biological matrices including nutraceutical products and more specifically to investigate the similarities and differences that the sugar moiety has on the pharmacokinetic disposition of these compounds and in particular their urinary excretion patterns.

Biomedical literature is devoid of validated assays for the detection of β-glucogallin. However, detection of gallic acid in a variety of assays has previously been reported in enzymology studies. Gallic acid has been detected using HPLC coupled to a variety of detection techniques including UV, diode array and evaporative light scattering detection [11–14]. To our knowledge there are no published studies that have developed and validated an analytical method for the detection of β-glucogallin in the literature and there are no species studies on the pharmacokinetic disposition of β-glucogallin. The aim of this study is develop an analytical method to simultaneously detect β-glucogallin and gallic acid and apply this developed method to urine samples. As β-glucogallin differs from gallic acid by a sugar moiety; a method that can simultaneously measure both β-glucogallin and gallic acid is of utility in pharmacokinetic studies in animals and disposition studies in plants.

There is a paucity of pharmacokinetic investigations with β-glucogallin and gallic acid. These current studies will examine feasibility of pharmacokinetic analysis of these compounds for urinary excretion studies in a rat model.

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doi:10.1016/j.jpba.2010.11.003

2. Experimental

2.1. Chemicals and reagents

β-Glucogallin was a generous gift provided by the Sabinsa Corporation® (Piscataway, NJ, USA). Gallic acid and 3-morpholin-4-ylpropane-1-sulfonic acid (MOPS), was purchased from Sigma–Aldrich® (St. Louis, MO, USA). HPLC grade methanol, water, and formic acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). High-pressure liquid nitrogen gas and compressed nitrogen gas were purchased from Central Stores (Washington State University, Pullman, WA, USA). Male Sprague-Dawley rats were obtained from Simonsen, (Gilroy, CA, USA).

2.2. Chromatographic system and conditions

The Shimadzu LC/MS system (Kyoto, Japan) consisted of a LC-10AD pump, a SIL-10AF auto-injector, a SPD-10AVP UV-VIS detector, a SCL-10AVP system controller, and a LCMS-2010 EV liquid chromatograph mass spectrometer. The mass spectrometer parameters consisted of a curved desolvation line (CDL) and block temperatures of 250 °C, the CDL, interface, and detector voltages at −8.0 kV, 4.5 kV, and 1.5 kV, respectively, and vacuum maintained by an Edwards® E2M30 rotary vacuum pump (Edwards, UK). Nebulizing (1.5 L/min) and drying gas (0.1 L/min) was supplied by high-pressure liquid nitrogen. The conditions for this method utilizes a Phenomenex® Luna® $C_{18}(2)$ column (4.6 mm × 250 mm, particle size 5 μm, pore size 100 Å; Phenomenex®, Torrance, CA, USA). The mobile phase consists of a mixture of water, methanol, and formic acid (90:10:0.1, v/v/v) (pH 3.2), modified with 2 mM ammonium acetate was filtered and degassed prior to use, with a flow rate of 0.7 mL/min. UV detection was monitored at 285 nm while β-glucogallin (*m*/*z* 331.25) and gallic acid (*m*/*z* 169.10) was monitored in the negative selected ion mode (SIM) and MOPS (internal standard) (*m*/*z* 210.25) was monitored in the positive SIM.

2.3. Stock and working solutions

Stock solutions of β-glucogallin, gallic acid, and MOPS maintained in HPLC grade methanol at a concentration of 100 μg/mL were protected from light, and stored at −20 °C for no more than 3 months. Working solutions were prepared at concentrations of 1.0, 5.0, 10.0, 50.0, and 100.0 μg/mL (working standards) for each compound in methanol. The peak absorbances for each of the stock solutions were determined spectrophotometrically using a spectrophotometer (Shimadzu UV2100U, UV-VIS recording spectrophotometer) data were collected using the UV-2101PC software.

2.4. Animals

Male Sprague/Dawley rats were placed in metabolic cages with *ad libitum* access to food (8664, Harlan Teklad F6 Rodent Diet, Madison, WI, USA) and water. Urine was collected and pooled into a sterile container and stored at −20 °C. This urine was used as the blank male rat urine throughout the study. This urine was free of any interfering peaks at the retention times of the internal standard (IS), β-glucogallin, and gallic acid; additionally, β-glucogallin and gallic acid were not quantifiable in the collected blank urine. The use of animals in this study was approved by the Institutional Animal Care and Use Committee at Washington State University.

2.5. Sample preparation

Standard curves were prepared with blank male rat urine (0.10 mL), IS (0.050 mL), and working standards (0.10 mL) combined in 2.0 mL microcentrifuge tubes (VWR International, West Chester, PA, USA). Precipitation of proteins and extraction of β-glucogallin, gallic acid, and MOPS was accomplished with 10% formic acid in water (1 mL), vortexed (Vortex Genie-2, VWR Scientific, West Chester, PA, USA) for 90 s, incubated in a hot water bath (60 °F) with sonication (Cole-Palmer® 8893, Vernon, Hills, IL, USA) for 30 min, centrifuged (Beckman® J-6B, Brea, CA, USA) for 5 min at 5000 rpm at 0 °C. The supernatants were collected and dried using a SpeedVac® (Savant SC110 A SpeedVac® Plus Concentrator with Universal Vacuum System (UVS400), Savant Instruments, Inc, Holbrook, NY, USA). The dried working standards were reconstituted in mobile phase (0.20 mL), centrifuged for 5 min at 10,000 rpm, transferred to HPLC vials (SUN SRi Rockwood, TN, USA), and injected (0.050 mL) into the HPLC/ESI/MS system. Unknown quality control and pharmacokinetics samples were prepared using the same method of extraction as the standard curves.

2.6. Validation

In the development of a method, a multitude of conditions are evaluated such as sample processing, mobile phase pH, retention time, flow rate, and column. The validation of a method is critical as future applications are dependent on the reproducibility of the method within regulated guidelines set forth by the International Conference on Harmonization (ICH) as published by agencies like the Food and Drug Administration [15]. This method was validated in urine over the working standard range using the aforementioned processing, using ICH criteria as a guideline.

2.6.1. Precision and accuracy

Precision and accuracy were evaluated by the relative standard deviation (RDS) and mean percentage error of measured concentration to the actual concentration (bias) over the working standard concentration range ($n = 6$), respectively. The within-run precision and accuracy of the method were determined using the same samples injected into the HPLC/ESI/MS system on the same day ($n = 6$). The between-run precision and accuracy of the method over the working standard range were determined in replicate ($n = 6$) using samples that were prepared on different days in the same way; thus samples were injected into the HPLC/ESI/MS system on different days within 1 week. The matrix effects were evaluated by comparing the peak area obtained from the post-extraction spiked working standard samples to a pure solution sample at the same nominal concentrations.

2.6.2. Linearity, LLOQ, and LLOD

A minimum of five points were used in the validation of this assay. The lower limit of detection (LLOD) is the lowest detectable concentration that is differentiable from noise produced by chromatography instrumentation. The lowest concentration of the standard curve that falls within ICH criteria which states that the RSD and bias must fall within 20% of the theoretical value is identified as the LLOQ. Additionally, all other points must fall within 15% of the theoretical value. Linearity is evaluated with lines obtained from standard curves having coefficients of correlation of ≥ 0.95 were considered acceptable along with standard deviations of slope and intercept were utilized to describe linearity.

2.6.3. Recovery

The recovery of β-glucogallin and gallic acid was determined over the working standard range. Two sets of samples were prepared as in the standard curves section without extraction and with extraction and injected into the HPLC/ESI/MS system. The closer to 100% the extracted IS, β-glucogallin, and gallic acid urine peak area are to the unextracted peak areas the lack of deleterious effects the extraction process has on the samples.

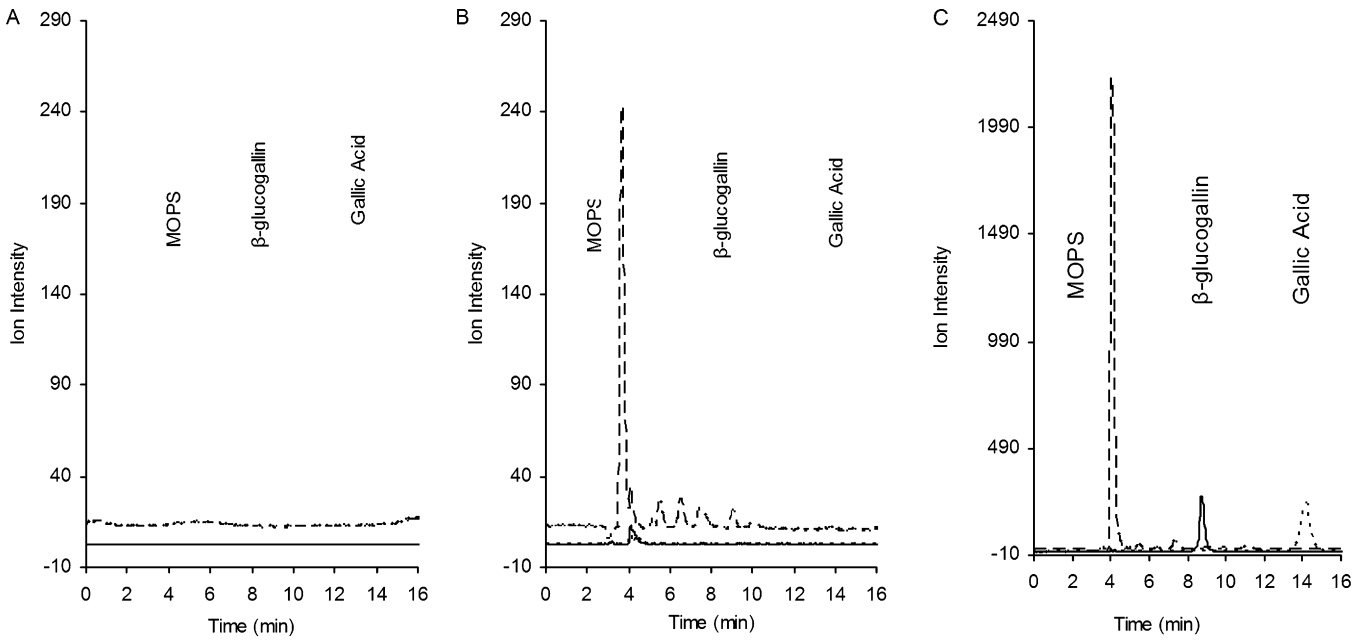

Fig. 1. Representative chromatogram using ESI/MS in the selected ion mode (SIM), ionization in the negative (β-glucogallin and gallic acid) and positive (MOPS) ion mode. MOPS (large dash), β-glucogallin (solid), and gallic acid (small dashes), blank (A), internal standard spiked in blank urine (B), and at 100 μg/mL in urine (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.6.4. Stability

The bench top stability of β-glucogallin and gallic acid was examined by preparing working standards as previously described. Samples were prepared on the first day, injected into the HPLC/ESI/MS system in replicate ($n = 3$), left overnight in the auto-injector rack for 24 h at ambient temperature and injected into the HPLC/ESI/MS system. The freeze–thaw stability of β-glucogallin and gallic acid was examined starting with powder to make new methanolic stock solutions and sample standard curves were prepared as previously stated. Samples were injected into the HPLC/ESI/MS system in replicate assays without being frozen, and then stored at −20 °C in capped HPLC vials and thawed at room temperature for one cycle as these samples were only thawed once.

2.7. Data analysis

Data collection and integrations were carried out using LCMS Solution software (Shimadzu, Kyoto, Japan). Microsoft® Excel will also used for data analysis. Each chromatogram from a sample consisted of three peaks: IS, β-glucogallin, and gallic acid. Under each peak is an area that can be quantified upon integration. The peak area ratio (PAR) is obtained by dividing the area of the β-glucogallin or gallic acid by the area of the area of the IS. The quantification of concentrations is calculated through unweighted least squares linear regression obtained from standard curves ($n = 6$) for both β-glucogallin and gallic acid. In the linear equation $y = mx + b$, y is the PAR, m is the slope, x is the concentration, b is the y-intercept, the standard curves from the validated assays determined the slope and y-intercept.

2.8. Pharmacokinetic analysis

From the concentrations experimentally determined, the pharmaceutical industry standard pharmacokinetic and pharmacodynamic modeling software WinNonlin® Version 1.0 was used. Data was modeled using non-compartmental analysis model 210 used for the analysis of urine data after an extravascular input. Additionally, modeled parameters were verified manually for urinary elimination rate constant (K) and fraction excreted unchanged in urine (Fe) using the following equations for the $K = 0.693/t_{1/2}$, where $t_{1/2}$ is the half-life and $Fe = \sum Xu/\text{dose}$, where Xu is the sum of the amount excreted in urine.

3. Results and discussion

3.1. Chromatography

Method conditions including pH, mobile phase, and column, the performance of the assay was assessed by examining peak shape and purity, interference from endogenous substances in biological fluids, linearity, LLOD, LLOQ, stability of reconstituted extracts, precision, accuracy, recovery, and matrix effect. These parameters were examined to achieve the most suitable conditions to achieve the simultaneous elution of β-glucogallin and gallic acid. An isocratic method was developed for the simultaneous detection of β-glucogallin, and gallic acid. Under these validated conditions, the IS, β-glucogallin, and gallic acid elutes at ~4, 9, and 14 min respectively at ambient temperature with UV detection set at 285 nm with no interfering peaks co-eluting with the compounds of interest and the IS. Detection was accomplished using the MS in the negative selected ion mode (SIM) for β-glucogallin (m/z 331.25) and gallic acid (m/z 169.10) or in the positive SIM for the internal standard MOPS (m/z 210.25) (Fig. 1). Use of the negative SIM mode for MOPS is also feasible.

Evaluation of possible IS were screened by first examining the distribution ratio ($X \log P$). Ideally, structurally and physicochemically similar compounds are selected as internal standards, however in this case, many of those similar compounds were present as metabolites and/or in botanicals. MOPS which is readily commercially available was selected because of suitable difference in retention times from the solvent front, as well as β-glucogallin and gallic acid, which allowed for good baseline separation between all three compounds and suitable peak shape and purity and lack of interference from endogenous substances.

Table 1
Validation of the developed method. Intra- and inter-day precision and accuracy and recovery of the assay for β-glucogallin and gallic acid in rat urine. Data is presented as mean ± SD.

Concentration (μg/mL)	Observed				RSD (%)				Bias (%)				Recovery (%)	
	Intra-day		Inter-day		Intra-day		Inter-day		Intra-day		Inter-day		β-Glucogallin	Gallic acid
	β-Glucogallin	Gallic acid	β-Glucogallin	Gallic acid	β-Glucogallin	Gallic acid	β-Glucogallin	Gallic acid	β-Glucogallin	Gallic acid	β-Glucogallin	Gallic acid		
1	0.99 ± 0.08	0.95 ± 0.08	0.81 ± 0.28	1.08 ± 0.05	18.83	19.09	1.6	12.73	−1.38	−5.14	−20.53	8.71	83.80 ± 0.09	92.28 ± 0.06
5	4.69 ± 0.14	5.03 ± 0.12	5.11 ± 0.26	5.00 ± 0.18	6.49	5.2	0.27	12.23	−6.11	0.72	2.29	0.03	97.40 ± 0.09	109.53 ± 0.35
10	10.24 ± 0.30	10.11 ± 0.38	9.99 ± 0.52	10.45 ± 0.30	6.53	8.45	0.27	8.24	2.44	1.09	−0.13	4.54	114.96 ± 1.05	95.32 ± 0.34
50	49.99 ± 0.46	49.93 ± 0.80	51.81 ± 1.66	51.11 ± 0.56	2.25	5.07	0.16	3.98	−0.018	−0.13	3.63	2.22	105.29 ± 1.94	109.23 ± 3.34
100	98.98 ± 2.62	100.13 ± 1.29	99.67 ± 1.16	99.89 ± 0.60	5.93	2.87	0.052	2.69	−1.02	0.13	−0.32	−0.11	98.16 ± 1.29	95.56 ± 3.06

The use of UV detection was not sufficient in the biological matrices as there were other peaks that resolve near the IS and β-glucogallin. Greater detection was not observed through MS than UV. The MS was able to accurately, consistently, and clearly identify and differentiate the IS, β-glucogallin, and gallic acid in biological samples.

3.2. Validation

3.2.1. Linearity, LLOQ, and LLOD

A linear relationship for β-glucogallin and gallic acid (average, $R^2 = 0.996$ and $R^2 = 0.997$, respectively) was established over the working standard concentration range as established by the concentration-PAR values. Additionally, linearity can be described as slope (±SD) are 0.0081 (±0.0034) and 0.0057 (±0.0019) for β-glucogallin and gallic acid, respectively; while the intercept (±SD) are 0.021 (±0.014) and 0.010 (±0.0052). The LLOQ for these compounds are 1 μg/mL corresponding to an RSD and a bias <20% less than 20%; with a LLOD 0.05 μg/mL (Table 1).

3.2.2. Recovery and bench top stability

The mean extraction efficiency for β-glucogallin from rat urine varied from 83.80% to 114.96% while the mean extraction efficiency for gallic acid from rat urine varied from 92.28% to 109.53%. Comparable peak areas were observed indicative of a lack of significant degradation of β-glucogallin or gallic acid in the bench top stabil-

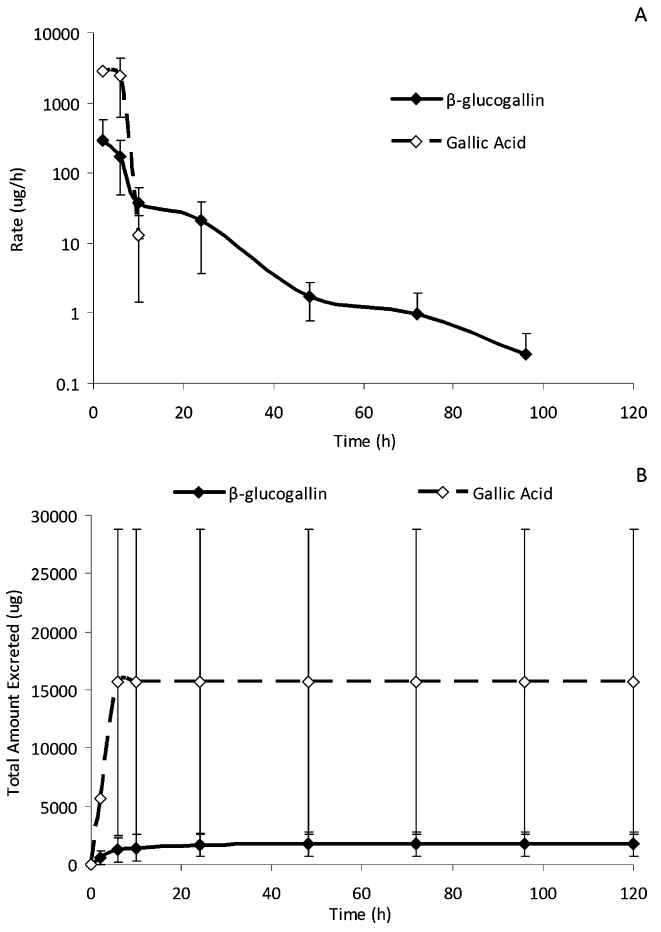

Fig. 2. (A) Cumulative urinary excretion of β-glucogallin and gallic acid, from rats ($n = 3$) after an oral dose 100 mg/kg of β-glucogallin. (B) Urinary excretion rate plot of β-glucogallin and gallic acid, from rats ($n = 3$) after an oral dose 100 mg/kg of β-glucogallin.

ity samples over the 24-h period and after a freeze–thaw cycle. Matrix effect was calculated as ME (%) = [(post-extraction − pure solution)/pure solution] × 100. In this study a comparison between the peak areas of the samples that were processed without processing and with processing were used to determine the effect of the matrix on the sample. It was determined that the variance in the ions was 9.04% for β-glucogallin and −6.03 for gallic acid at the LLOQ.

Decomposition of β-glucogallin by the ion source (ESI) was not observed as an aglycone mass, in this case, gallic acid was not detected in parallel with β-glucogallin from a sample initially void of gallic acid. A summary of the intra- and inter-day bias, RSD, and recovery is presented in Table 1.

The applicability of this analytical assay to determine the rate of urinary excretion in rats is demonstrated in (Fig. 2A). This HPLC/ESI/MS method was effectively applied to urine samples collected over time in rats dosed orally with β-glucogallin. Fig. 2A demonstrates that the rates of urinary excretion for β-glucogallin and gallic acid and are not identical as indicated by their elimination slopes. It is also evident that β-glucogallin was metabolized into gallic acid as rats were not administered gallic acid. Fig. 2B shows the cumulative urinary excretion rate plot over time. This figure shows that it is primarily the intact β-glucogallin excreted in urine with the formation of small amounts of gallic acid also occurring.

Preliminary pharmacokinetic parameters were delineated using WinNonlin®. The urinary half-lives for β-glucogallin was ∼31 h with elimination rate constants of ∼0.025 1/h. The fraction of β-glucogallin excreted unchanged in urine was ∼8% of the dose.

4. Conclusions

In summary, a novel, validated, reproducible, accurate HPLC/ESI/MS method was developed for simultaneous detection and quantification of β-glucogallin and gallic acid in urine in rats administered β-glucogallin. Further studies are being conducted in our laboratory to further characterize the content uniformity of nutraceuticals and the presence of these tannins in various plant samples reported to contain β-glucogallin and tissue and metabolite disposition pharmacokinetics and pharmacodynamics of β-glucogallin and gallic acid.

Acknowledgment

The authors would like to acknowledge an unrestricted grant from the Sabinsa Corporation®.

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