Original Article

A validated stability-indicating HPLC method for analysis of glabridin prodrugs in hydrolysis studies

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ABSTRACT: A simple, selective and precise stability-indicating HPLC method for determination of glabridin diacetate and dihexanoate prodrugs was developed, validated and applied to the enzymatic and chemical hydrolysis studies. The chromatographic separation was achieved on a reverse phase C18 (Thermo Hypersil-Keystone, 250 × 4.6 mm, 5 micron) column using the mixture of acetonitrile and water as mobile phase. Elution of the mobile phase was operated on isocratic (acetonitrile 76%: water 24%) for 9 min, followed by gradient (acetonitrile from 76% to 90%) within 9 min and isocratic (acetonitrile 90%: water 10%) for 12 min at 1 mL/min flow rate, detected at 280 nm. The method was validated for specificity, accuracy, precision, linearity and limit of quantitation following the International Conference on Harmonization (ICH) guidelines. The method is effective for the separation of glabridin diacetate and glabridin dihexanoate from glabridin, its parent drug and successfully used in these prodrugs hydrolysis studies.

Keywords: Glabridin, prodrugs, HPLC, validation, hydrolysis studies

1. Introduction

Glabridin is a major pyranoisoflavan isolated from hydrophobic fraction of European licorice, *Glycyrrhiza glabra* L.var *typica* and var *glandulifera* (*1*). It has been reported to exhibit a wide range of pharmacological activities such as antimicrobial (*2-4*), protection of mitocondrial functions against oxidative stress (*5*), estrogenic and antiproliferative activities in human breast cancer cells (*6,7*), inhibition of adenosine 3',5'-cyclic monophosphate (cAMP) phosphodiesterase (*8*), inhibition of human cytochrome P450s 3A4, 2B6 and 2C9 (*9*), inhibit melanogenesis and inflammation (*10*), neuroprotective (*11*) and prevention of low-density lipoprotein oxidation (*12-16*).

Even though glabridin (G) is a potent tyrosinase inhibitor and potential for skin whitening preparation, G showed poor skin penetration and instability in formulation (*15*). One strategy to overcome this problem is the prodrug approach. To improve the stability in formulation, glabridin diacetate (GDA) and glabridin dihexanoate (GDH) esters were synthesized by acylation as prodrug of G (Figure 1). A successful G prodrug must not only undergo enzymatic hydrolysis in physiological condition, but also be chemically stable in formulation. So far, to our present knowledge, most previous analytical methods for determination of G (*16-20*) have been done in licorice and its extract, but no stability-indicating analytical method for the quantitation of G prodrugs was available in literature. Therefore, it is necessary to develop an analytical method to determine the G, GDA and GDH in hydrolysis studies.

This paper deals with the HPLC assay and method validation for accurate quantitation of G, GDA and GDH. The paper also deals with the application of the method to enzymatic and chemical hydrolysis studies.

2. Materials and Methods

2.1. *Chemicals*

G was isolated from available licorice extract (PT-40) by conventional column chromatography and obtained

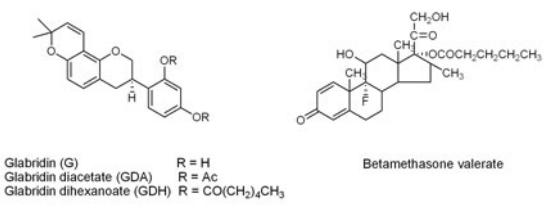

Figure 1. Structure of glabridin (G), its prodrugs and betamethasone valerate (IS).

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G was finally purified by crystallization from hexane. GDA and GDH were synthesized by acylation with corresponding acid anhydrides. The GDA was purified by column chromatography and recrystallized from methanol, while GDH was purified by column chromatography. The structures of products were elucidated by spectroscopic techniques, mainly NMR, MS, IR spectroscopy and elemental analysis as well as comparison with those of earlier reported ^{1}H and ^{13}C NMR data of G (*21*). G, GDA and GDH purity were determined based on HPLC peak purity analysis.

HPLC grade acetonitrile was purchased from Lab-Scan Analytical Sciences (Bangkok, Thailand). Water was purified using a ELGA water purifier (ELGA Ltd., Bucks, England). All other chemicals and solvents were analytical grade.

2.2. *Equipment*

The quantitation of G, GDA, and GDH was performed on a Shimadzu HPLC system (Kyoto, Japan) consisting of SCL-10ADvp system controller, two LC-10ADvp pumps, SIL-10ADvp autosampler, Diode-array detector SPD-M10Avp, and Class VP software. Shaking water-bath (Maxi shake SBD-50 COLD, Allerod, Denmark), Centrifuge (Hettich Zentrifugen EBA-20, Tuttingen, Germany) and Vortex-2 Genie (Scientific industries, Inc., Bohemia, USA) were used for hydrolysis studies.

2.3. *Standard solutions preparation*

Each stock solution of G (0.15 mg/mL), GDA (0.20 mg/mL), GDH (0.19 mg/mL) and internal standard (IS), betamethasone valerate (0.41 mg/mL) was prepared in acetonitrile. Working standard solutions for calibration curve were prepared by diluted of each stock solution with acetonitrile to give the final concentration range of 0.075-13.5 µg/mL for G, 0.1-20.0 µg/mL for GDA, and 0.95-16.15 µg/mL for GDH, respectively. The IS solution was added to each working standard solution to obtain the final concentration of 82 µg/mL.

2.4. *Sample preparation*

The stock solutions (0.01 M) of each G, GDA, and GDH were prepared in dimethylsulfoxide and IS solution (82 µg/mL) was prepared in acetonitrile. For determination of extraction recovery and precision, 50 µL of the standard solution was added to 450 µL of phosphate buffer solutions (pH 5.5 and 7.4) in a 10-mL screw-capped test tube. One mL of methanol was added and vortexed for 3 sec. The extract was performed by adding 3 mL of chloroform. After 10 sec vortexing, the mixture was centrifuged at 5,000 rpm for 5 min. The aqueous layer was removed and the organic phase was transferred to a new test tube. Two mL of organic phase was evaporated to dryness and reconstituted in 1 mL of IS solution, then filtered through 0.45 µm nylon filter to obtain a clear sample solution.

2.5. *Chromatographic conditions*

A reverse phase C18 (250 × 4.6 mm, 5 micron; Thermo Hypersil-Keystone, Bellefonte, PA, USA) column connected with a guard column (C18; Alltech Associates, Inc., Deerfield, IL, USA) was used. The mobile phase was a mixture of acetonitrile and water at 1 mL/min of flow rate. To separate the sample, three steps of the elution was operated, isocratic elution with the mixture of acetonitrile and water (76:24, v/v) for 9 min followed by linear gradient elution of 76% to 90% acetonitrile for 9 min and finally isocratic elution with the mixture of acetonitrile and water (90:10, v/v) for 12 min. The eluent was monitored at 280 nm. The injection volume was 20 µL of sample solutions.

2.6. *Method validation*

The validation was performed by spiked placebo technique. The method was validated for specificity, accuracy, precision, linearity and limit of quantitation was determined in accordance with ICH guidelines on analytical validation Q2 (R1) (*22*).

2.6.1. *Specificity*

Specificity is the ability of the method to measure the analyte in the presence of impurities and degradation products (*22*). The specificity of the developed HPLC method for the prodrugs, GDA and GDH were determined by the retention time of GDA, GDH and G in the HPLC system. In addition, the peak purity tests of each standard were performed by the photodiode array detection.

2.6.2. *Linearity and range*

The linearity of the method was conducted by preparing standard solutions at five different concentrations of analytes within range 0.75-13.5, 0.1-20.0 and 0.95-16.15 µg/mL for G, GDA, and GDH, respectively. Each solution was analyzed in triplicate. Peak area ratio values (ratio AUC/IS) were plotted against the corresponding concentrations of analytes. The linearity of the plot was evaluated by linear regression analysis.

2.6.3. *Accuracy*

The accuracy of the calibration standards was evaluated in triplicate at three concentrations (4.50, 7.50, and 10.50 µg/mL for G, 6.00, 10.00, and 14.00 µg/mL for GDA and 1.90, 7.60, and 11.40 µg/mL for GDH). To access the accuracy of the samples in spiked phosphate buffer systems, three different concentration levels

(6.49, 8.65, and 10.81 µg/mL for G, 9.73, 12.97, and 16.22 µg/mL for GDA and 9.41, 12.54, and 15.68 µg/mL for GDH) were prepared as described above and injected to HPLC. The percentage of recoveries was calculated from slope and y-intercept of the calibration curve. The criteria for acceptability of accuracy or recovery were the average value within 95-105%.

2.6.4. *Precision*

The intra-day and inter-day precision of both calibration standards and samples were evaluated at the same concentration levels as those performing for the accuracy. The intra-day precision was performed on the same day and expressed as percentages of relative standard deviations (%RSD) calculated from the analytes of three replicates samples. The inter-day precision was performed on three separate days. The criteria for acceptability of precision was the %RSD for each concentration level not exceed 3%.

2.6.5. *Limit of quantitation*

The limit of quantitation (LOQ) of the analytes was determined during the evaluation of the linear range of the method. The LOQ was defined as the lowest concentrations yielding a precision with RSD ≤ 10% and acceptable accuracy within 10% of the theoretical value.

2.7. *Enzymatic and chemical stabilities*

For the enzymatic hydrolysis studies, a purified porcine liver esterase (PLE, carboxylic-ester hydrolase, EC 3.1.1.1; Sigma-Aldrich, St Louis, MO, USA) was dissolved in 0.05 M phosphate buffer solution (pH 7.4, ionic strength = 0.15) to a concentration of 1.01 unit/mL (*23,24*). Eighty microliters of the 0.01 M stock solution of the prodrug solution in dimethylsulfoxide was added to the enzyme-buffer mixture, mixed well and then kept in a water-bath at 37 ± 0.1ºC. At appropriate time intervals (0, 2, 4, 6, 8, 10, 15, 30, 45, 60, 90, and 120 min for GDA and 0, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 31 h for GDH), 500 µL aliquots were withdrawn and 1 mL of methanol was added to quench the reaction. The extract was performed as described in sample preparation. Pseudo-first-order rate constants (k) for the hydrolysis were determined from the slopes of linear plots of the logarithm of residual model prodrugs against time. The half-life of each prodrug was calculated from the equation: $t_{1/2} = 0.693/k$. The experiments were performed in triplicate.

The chemical stability of the prodrug was investigated in aqueous phosphate buffer solutions (pH 7.4 and 5.5, ionic strength = 0.15) at 37 ± 0.5°C, in triplicate experiments. At 24-hour intervals, sample was taken, extracted as the method described above and analyzed by HPLC.

3. Results

3.1. *Separation and specification*

The developed method showed well chromatographic separation with no interference at the elution time of analytes. Chromatograms of G and its prodrugs sample spiked with IS were recorded at 280 nm according to their absorption maxima as shown in Figure 2. The retention time in HPLC condition were 4.70, 5.27, 7.76, and 25.04 min for G, IS, GDA, and GDH, respectively.

3.2. *Linearity and range*

Linear calibration plot was obtained from the theoretical concentration versus peak area response within the range of 0.075-13.50, 0.10-20.0, and 0.95-16.15 µg/mL for G, GDA, and GDH, respectively. Response linearity was determined by least squares regression analyses of the calibration plot. The linear regression (r^2) of calibration plots of G, GDA, and GDH showed good linear relationship with r^2 = 0.9998, 0.9999, and 0.9999 with respect to peak area (Table 1).

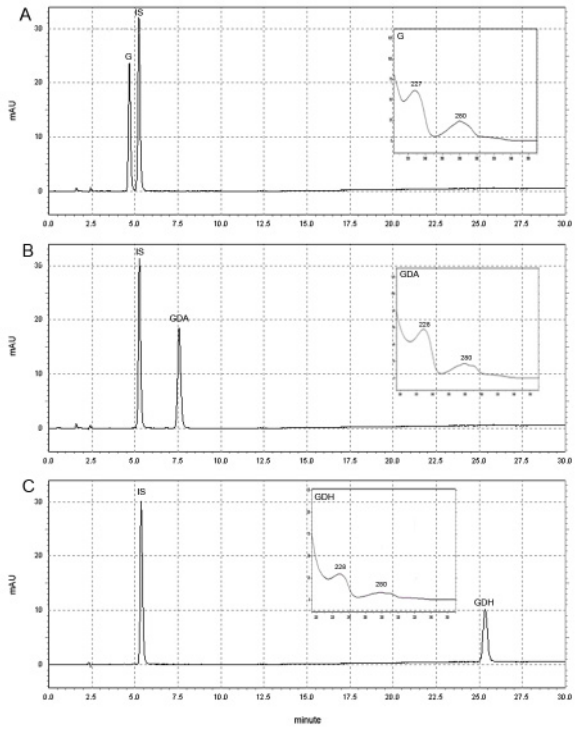

Figure 2. HPLC chromatograms and photodiode array UV absorption spectra of (A) G 4.5 µg/mL, (B) GDA 10 µg/mL and (C) GDH 7.6 µg/mL, spiked with IS 82 µg/mL.

Table 1. Regression data of calibration curves of each reference standard

Analyte	Linear regression	Correlation coefficient (r^2)	n
G	y = 0.1535x – 0.0023	0.9998	3
GDA	y = 0.0818x + 0.0004	0.9999	3
GDH	y = 0.0652x + 0.0226	0.9999	3

3.3. *Accuracy and precision*

The accuracy and repeatability of samples at three concentration levels were expressed in terms of % recovery and %RSD. The accuracy and precision of the quantitation of calibration standard were shown in Table 2. The assay exhibits acceptable level of accuracy for estimating analyte concentration (95-105%) within percent relative standard deviations (%RSD) < 3%. The percent recovery of the standards ranged from 98.86-101.1%, 98.44-99.74%, and 100.1-102.8% for G, GDA, and GDH, respectively, with precision < 3%. Similar level of accuracy and precision were obtained from the analysis of the samples in both buffer systems (Tables 3 and 4).

3.4. *Limit of quantitation*

Limit of quantitation (LOQ) is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. LOQ was determined during the evaluation of the linear range of calibration curve and defined as the lowest concentration yielding a precision with %RSD less than 10, accuracy within 10% of the

Table 2. Accuracy and precision of the quantitation of calibration standard (*n* = 3)

Analyte	Added (μg/mL)	Intra-day			Inter-day		
		Found (μg/mL)	Recovery (%)	RSD (%)	Found (μg/mL)	Recovery (%)	RSD (%)
G	4.50	4.45	98.86	1.20	4.45	98.84	0.16
	7.50	7.50	100.0	1.05	7.43	99.04	0.90
	10.50	10.61	101.1	0.07	10.38	98.87	1.93
GDA	6.00	5.91	98.44	0.38	5.99	98.87	1.48
	10.00	9.91	99.08	0.93	9.84	98.42	0.65
	14.00	13.96	99.74	1.09	13.95	99.65	0.46
GDH	1.90	1.95	102.8	1.85	1.94	102.0	1.07
	7.60	7.65	100.7	0.51	7.79	102.6	1.60
	11.40	11.41	100.1	1.82	11.56	101.4	2.27

Table 3. Intra-day precision of the quantitation of sample in spiked phosphate buffer systems (*n* = 3)

Analyte	Added (μg/mL)	pH 5.5			pH 7.4		
		Found (μg/mL)	Recovery (%)	RSD (%)	Found (μg/mL)	Recovery (%)	RSD (%)
G	6.49	6.41	98.75	2.43	6.55	101.0	1.89
	8.65	8.39	97.06	1.17	8.55	98.81	1.49
	10.81	10.88	100.6	1.60	11.03	102.1	2.10
GDA	9.73	9.68	99.50	1.33	9.43	96.90	0.56
	12.97	12.73	98.10	0.67	13.10	101.0	2.26
	16.22	16.43	101.3	1.35	16.04	98.94	0.25
GDH	9.41	9.45	100.5	1.52	9.55	101.6	2.75
	12.54	12.70	101.3	2.53	12.57	100.2	1.24
	15.68	15.55	99.17	1.01	15.53	99.06	1.83

Table 4. Inter-day precision of the quantitation of sample in spiked phosphate buffer systems (*n* = 3)

Analyte	Added (μg/mL)	pH 5.5			pH 7.4		
		Found (μg/mL)	Recovery (%)	RSD (%)	Found (μg/mL)	Recovery (%)	RSD (%)
G	6.49	6.40	98.61	0.49	6.57	101.4	0.54
	8.65	8.49	98.19	1.05	8.63	99.76	1.60
	10.81	11.06	102.3	1.54	10.83	100.2	1.59
GDA	9.73	9.83	101.0	1.79	9.40	96.90	0.52
	12.97	12.66	97.62	1.78	12.86	99.14	2.15
	16.22	16.38	101.0	0.75	16.21	99.94	1.13
GDH	9.41	9.53	101.3	2.05	9.56	101.7	2.23
	12.54	12.68	101.2	0.29	12.55	100.1	1.18
	15.68	15.58	99.37	0.49	15.63	99.73	1.16

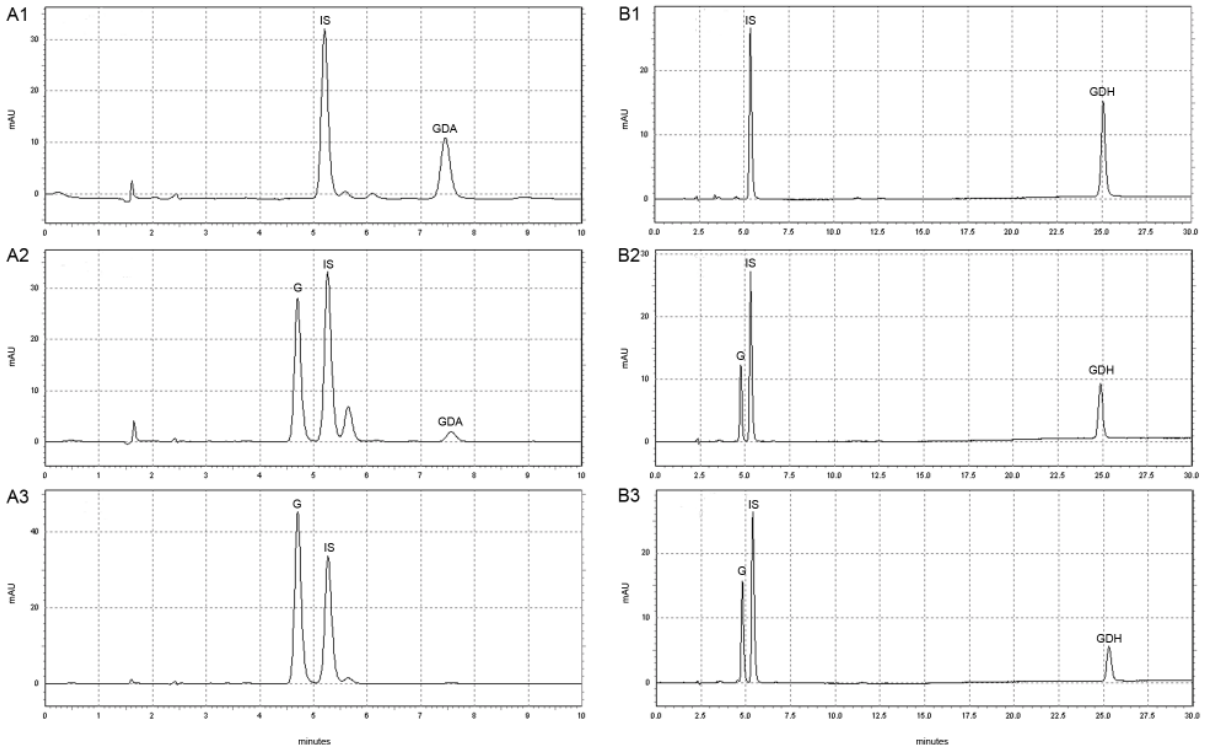

Figure 3. HPLC chromatograms of samples from enzymatic hydrolysis studies of GDA (A) at 0 min (A1), 6 min (A2), and 120 min (A3) as well as GDH (B) at 0 h (B1), 10 h (B2) and 24 h (B3).

theoretical value (90-110%R). The established LOQ were 0.075, 0.1, and 0.95 µg/mL for G, GDA, and GDH, respectively.

3.5. *Application to enzymatic and chemical stability studies*

The validated method was further evaluated for its applicability in analyzing G, GDA, and GDH in stability studies. In this study, the porcine esterase was used as a tool for enzymatic hydrolysis study of G prodrugs and the chemical hydrolysis was determined in phosphate buffer (pH 5.5 and 7.4). The chromatograms of analytes from enzymatic hydrolysis of GDA and GDH were shown in Figure 3 and chemical hydrolysis time course profiles of GDA and GDH were demonstrated in Figure 4. The enzymatic half-life of GDA was 2.36 min and that of GDH was 14.8 h, while chemical hydrolysis half-lives of both prodrugs were more than 15 days.

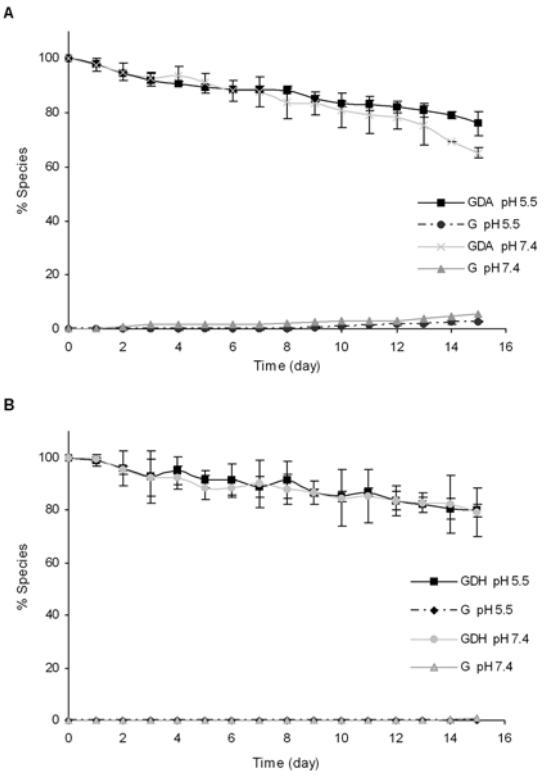

Figure 4. Chemical hydrolysis time course for GDA (A) and GDH (B) including its parent drug, G, in phosphate buffer systems.

4. Discussion

The main target of this chromatographic method is to develop a single HPLC system for the simultaneous separation of both GDA and GDH prodrugs from its parent drug. The developed method was based on different polarity, amenable to reverse-phase HPLC and UV detectable at 280 nm of each analyte. In addition, the liquid-liquid extraction was employed as sample preparation method.

Several compounds, quercetin, plumbagin, berberine, diclofenac, flurosemide, chloramphenicol,

propyl-paraben, prednisolone, and betamethasone valerate were tested as internal standard. The peaks of all selected compounds except betamethasone valerate were not completely separated from the peak of either G or its prodrugs. Only betamethasone valerate was well separated from G, GDA, and GDH, and thus it is used as internal standard.

Various combinations of organic modifiers such as acetonitrile, tetrahydrofuran as well as methanol and water were investigated. Besides, both isocratic and gradient HPLC elutions were also examined. Finally, the optimal condition was achieved using the mixture of acetonitrile and water as mobile phase, which operated on isocratic (acetonitrile 76%: water 24%) for 9 min, followed by gradient (acetonitrile from 76% to 90%) within 9 min and finally, isocratic (acetonitrile 90%: water 10%) for 12 min at 1 mL/min of flow rate. The linear regression analysis data for the calibration plots of G, GDA, and GDH showed good linear relationship with r^2 = 0.9998, 0.9999, and 0.9999 with respect to peak area, for the concentration range 0.075-13.50, 0.10-20, and 0.95-16.15 µg/mL, respectively. The accuracy of each analyte was determined to be within 5% (95-105%) with a relative standard deviation < 3%. Thus the developed sample preparation and liquid chromatographic condition were found to be specific for GDA and GDH prodrugs including their parent drug, G.

The validated method was also tested for its ability to support the quantitative analysis of GDA and GDH prodrugs in stability studies. Since the precondition of prodrug approach is that the prodrug can be converted to the parent drug in order to exert biological effects. The evaluation of enzymatic and chemical hydrolysis of these prodrugs was studied. As porcine liver esterase is a good model of the esterase found in the skin and, as such, is important in predicting the use of certain prodrugs as topical treatment. Chromatograms shown in Figure 3 indicated that the porcine liver esterase can easily catalyze hydrolysis of phenol ester of GDA prodrug, lead to the release of its parent drug, G. The hydrolysis of GDA was found to proceed in two steps reaction. Firstly, one of the ester moiety was hydrolyzed to yield the intermediate, G acetate which underwent spontaneous hydrolysis to G. While GDH was hydrolyzed with more slower rate. Therefore, the varying size of the acyl group from acetate (C2) group of GDA to hexanoate (C6) group of GDH may cause steric hindrance to the esterase. Moreover, the stability of prodrug against the spontaneous chemical hydrolysis is of importance, especially for the cosmetic products and topical formulation. The chemical hydrolysis in this study was performed to evaluate the effect of pH on degradation of G prodrugs. The result indicated that the chemical stabilities of both prodrugs at both pH were quite similar. However, the degradation rate was very slow as comparison with enzymatic hydrolysis. The half-lives of hydrolysis in both pH buffer solution were more than 15 days, suggesting the sufficient chemical stability.

5. Conclusion

A simple, sentitive, accurate and precise HPLC method was developed, validated and subsequently successfully applied to stability studies of glabridin prodrugs. The stabilities of these prodrugs in enzyme solution and phosphate buffer systems (pH 5.5 and 7.4) were examined by monitoring the remained level of prodrugs as well as the appearance of their degradation product, glabridin, under the experimental conditions. Therefore, the chromatographic condition and sample preparation method of this present study is suitable to conduct stability studies for glabridin prodrugs.

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