Chapter

A New HPLC-DAD Method for the Simultaneous Measurements of Glycyrrhizic Acid (GA) and Glabridin (GB) in Licorice (*Glycyrrhiza glabra* L.) Extract

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Abstract

Glycyrrhiza glabra L. (Licorice) is a plant with important applications in the fields of medicine, food and cosmetics due to the extracts obtained from its root and the chemicals contained in these extracts. Glycyrrhizic acid (GA), a saponin glycosid, is the main component that gives licorice its sweet taste. The ammonium salt of this compound is listed in the European Pharmacopeia and is the sweetest natural flavoring listed on the Food and Drug Administration (FDA). Its main known properties are its effective properties in the treatment of ulcers, bronchitis, sore throats, liver diseases and its anti-inflammatory, antibacterial and antiviral effects (especially hepatitis C and HIV treatment). Glabridin (GB), an isoflavan, is known for its cytotoxic, antimicrobial, estrogenic and anti-proliferative effects in breast cancer. It is also valuable for its antioxidant effect on melanogenesis, low-density lipoprotein (LDL) oxidation and mitochondria functions. Due to all these effects, licorice is widely used by healthcare professionals, doctors and the public. It is extremely important to determine the contents of the active ingredients in licorice root extracts. This work devised an easy-to-use, fast and economical HPLC-DAD technique to analyze this bivalent chemical in products containing licorice root extract. The developed method will shed light on future bioavailability studies and the determination of these chemicals in extracts.

Keywords: *Glycyrrhiza glabra*, glycyrrhizic acid, glabridin, HPLC-DAD, method validation

1. Introduction

Glycyrrhiza glabra L. (Licorice) is a perennial woody shrub and is distributed in many regions of the Mediterranean and Asia. These include Turkey, Italy, Spain, Russia, Syria, Iran, China and Afghanistan [1]. The plant has blue-purple flowers. Licorice is a perennial shrub from the Fabaceae family that can grow up to



Figure 1.
General view and hammered root image of Glycyrrhiza glabra *L.*

approximately 120–150 cm tall. The root of the licorice plant is 2.5 ± 0.5 cm in diameter and 15–20 cm long. **Figure 1** shows the general appearance of the *Glycyrrhiza glabra* L. plant. This distribution is observed in our country in the provinces of Gaziantep, Iğdır, Kars, Siirt, Bitlis, Diyarbakir, Kahramanmaraş, Muş, Samsun and Şanlıurfa.

Licorice extract is 50 times sweeter than sugar and contains a mixture of triterpenoid saponins (4–20%) and potassium and calcium salts of glycyrrhizic acid (GA), including larger amounts of GA. Licorice root has a long history of medicinal use in Europe and Asia dating back thousands of years. It has a wider use in Asian countries and is used as a sweetener and also for healing wounds [2]. The syrup of this root is used effectively in the treatment of peptic ulcer disease, constipation, cough, bronchitis, cold, diabetes, lung and kidney stones [3]. In addition, it has a wide range of pharmacological effects such as antiulcer, antidiabetic, antiviral, antioxidant, antiallergic, anticancer, antidepressant, expectorant, anti-inflammatory in colitis and memory enhancer [4]. In addition to all these, it has been explained in the literature that licorice has uses in the treatment of osteoarthritis [5], systemic lupus erythematosus (SLE) [6], malaria [7], tuberculosis [8], food poisoning [9] and chronic fatigue syndrome (CFS) [10]. Shakuyaku-kanzo-to, a herbal product containing licorice, increases fertility in women with polycystic ovary syndrome [11, 12]. However, licorice has proven uses in the treatment of prostate cancer [13] and in the treatment of eczema [14, 15].

Some examples of products or preparations using this extract are cough syrups, food supplements, chewing gum, beverages and candies [16]. Tobacco product manufacturers have reported using licorice root to flavor tobacco and sweeten smoke at different stages of production. Licorice adds a ripe, sweet and woody flavor to the smoke. Additionally, in the cosmetic industry, licorice root can be used as a natural skin brightener as an alternative to chemical skin brighteners such as hydroquinone. In addition, it has been shown to be very beneficial to skin health when used externally, as it causes intracellular fat and hyaluronan synthesis [17].

The ammonium salt of GA (**Figure 2**) is listed in the European Pharmacopeia and is the sweetest natural flavor listed on the Food and Drug Administration (FDA). Most pharmacological studies on licorice focus on GA and its aglycone glycyrrhetic acid. These two compounds have many comprehensive biological activities such as antiulcer,

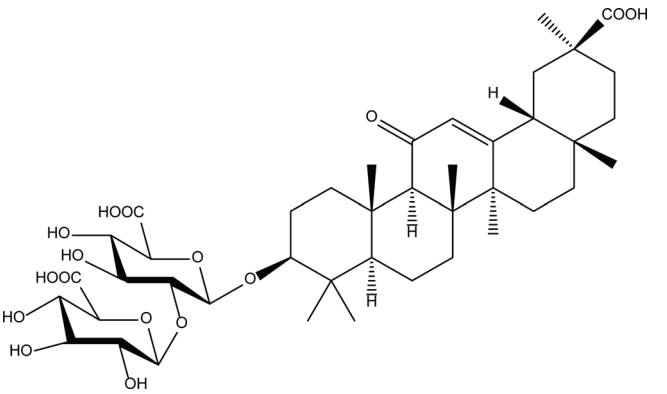


Figure 2.
Chemical structure of GA.

anti-inflammatory, antiallergic, antioxidant, anticarcinogenic, antithrombotic, antidiabetic and antiviral. Additionally, GA is used as a therapeutic agent against many viral diseases such as AIDS, and hepatitis B and C [18]. GA is a water-soluble pentacyclic triterpenoid glycoside that gives licorice its taste and is used as a sweetening additive and emulsifier in the food industry because it is 50 times sweeter than sugar. In many countries, GA is an important therapeutic agent used to treat allergic dermatitis and chronic viral hepatitis [2]. The acid form of GA is insoluble in water, but its ammonium salt is soluble in water (pH > 4.5). The monoammonium salt of GA is used as an anti-inflammatory, antiallergic agent for the treatment of bronchial asthma, eczema and other diseases [16]. In the cosmetic field, GA and stearyl glycyrrhetinate are used to reduce dryness and deformation in dry and deformed skin. It is also preferred after sun exposure due to its anti-inflammatory and soothing properties.

GB (**Figure 3**) has been reported to exhibit many pharmacological activities such as cytotoxic activity, antimicrobial activity and antiproliferative activity against breast cancer cells. Additionally, it is effective in protecting mitochondrial functions from melanogenesis, inflammation, low-density lipoprotein oxidation and oxidative stress [2]. It has been proven by research that GB significantly reduces inflammation in the intestine in colitis GB has an inhibitory effect on melanin production and inflammation [19]. It inhibits the formation of nitric oxide, a free radical. The use of GB at a concentration of 0.1% is sufficient to achieve the desired effect. It is also known to have an anti-inflammatory effect. It has a depigmentation effect that is 16 times stronger than hydroquinone, and no side effects have been detected in the long

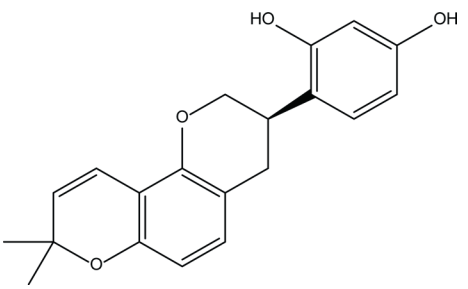


Figure 3.
Chemical structure of GB.

term [20]. GB is used as a whitening agent against age spots. GB helps heal red and scalded skin but is expensive for this treatment.

There are 2783 food supplements, cosmetic products or pharmaceutical formulations worldwide containing licorice extract or a pure compound containing this extract. While 116 of these contain high amounts of licorice, other products contain this extract as a sweetener or thickener in amounts up to 1% [21, 22]. FDA classifies licorice and its ingredients as generally recognized as safe (GRAS). This extract has not been described as toxic by any authority. When we look at the literature, there are very few methods for the determination of the components (GA and GB) responsible for the effect in products containing licorice extract (food supplements, cosmetics and drug formulations) using liquid chromatography. Current methods have been disadvantages due to the use of expensive detectors, long sample preparation procedures, and the small number of samples examined or the same type. Mass spectrometry is a unique analytical technique, but it is expensive when integrated with liquid chromatography. Additionally, in existing methods, the retention times of these two compounds (GA and GB) are quite long. Since the existing formulation in herbal extracts and food supplements is almost the same, there is no method that can determine such different types of formulations with a simple extraction method at low cost (UV detection) as in our study [23–25]. The aim of this study is to use body spray, cream, lotion, ointment, lozenge, nasal spray, nasal ointment, ear drops, soap and shampoo, in pharmaceutical products containing licorice. The goal is to create and verify an HPLC-DAD technique that will enable the simultaneous study of GB and GA without affecting other elements. The suggested approach will make it simple and quick to analyze pharmaceutical goods that are purported to contain GB, GA and licorice in standard laboratories.

2. Materials and methods

2.1 Chemicals and reagents

Acetonitrile, methanol, ethanol, chloroform, dimethylsulfoixde, triethylamine, glycyrrhizic acid and glabridin (≥95.0% HPLC) were acquired from Sigma-Aldrich. Merck provided the monobasic potassium phosphate (KH_2PO_4). About 0.45-μm polyethersulfone filters (Dainippon Seiki, Kyoto, Japan) were used for sample filtration. A human system (Japan) provided water purification.

2.2 Preparation of mobile phase and solutions

Monobasic potassium phosphate buffer (1.36 mg/mL) was included in mobile phase A. There was 100% acetonitrile in mobile phase B. As a diluent, 100% methanol was utilized. Making mixed standard solutions and stock solutions of glabridin and glycyrrhizic acid in order to create GA solutions of 0.1 mg/mL, 2.6 mg of the glycyrrhizic acid reference standard was dissolved in 25 mL of diluent, and 2.5 mg of the glabridin reference standard was dissolved in 25 mL of diluent. In order to create a mixed standard solution, 20 mL of methanol was used to dilute 2 mL of each standard stock solution—glabridin and glycyrrhizic acid—to get a concentration of 0.01 mg/mL for GA and GB. Working solutions were prepared by dilutions of various kinds. The stock standard and working solutions were kept +4°C and were remained stable throughout the research. For HPLC analysis, working standard solutions were

aliquoted in quantities of 30 μL. The chromatograms were analyzed using the peak regions versus concentration of the medicinal compounds.

2.3 Selection of wavelength

Spectrophotometric measurements were carried out using a Shimadzu UV-160 A spectrophotometer with 1-cm glass cells. The scanned UV spectra of the GA and GB solution, which ranged from 200 to 400 nm, were used to determine the detection of wavelength. The selected wavelength for GB was 280 nm, whereas the wavelength for GA was 254 nm, based on the UV spectrum.

2.4 Instrumentation and chromatographic conditions

The Shimadzu (Japan) liquid chromatography system, which included an LC-20 AT pump, SIL AT-HT autosampler component, SPD-20A HT DAD detector set at 254 nm and 280 nm, and CTO 10 AC column oven, was utilized for the HPLC analyses. To achieve the most effective chromatographic separation, a range of mobile phase, column types and stationary phase size combinations were tested with varying flow rates and column temperatures. On a GL Sciences (Japan) C18 (ODS) column with the dimensions of 4.6 mm I.D., 150 mm length and 5 μm particle size, chromatographic separation was achieved isocratically at 30°C. KH_2PO_4 makes up mobile phase A, while acetonitrile (100%), with a flow rate 1.3 mL/min, makes up mobile phase B.

2.5 Preparation of the calibration curves

A calibration curve was developed by examining working standard solutions of GA and GB at various concentrations. The linear concentration ranges of the method for the two medicines were calculated using linear least-squares regression (each concentration was examined as five repetitions). The calibration curve's equations have been obtained as $y = ax + b$, where x represents the drug substance concentrations in μg/mL and y represents the peak areas.

2.6 Application of the extraction process from the analyzed pharmaceutical products

Different extraction methods, such as liquid-liquid extraction (LLE) and solid phase extraction (SPE), are established for the purpose of extracting GA and GB out of pharmaceutical products. The liquid-liquid extraction (LLE) approach was initially investigated using a range of extraction solvents, combinations and extraction solvent volumes to create an efficient method of extracting GA and GB from pharmaceutical products employing the solid phase extraction (SPE) technique, and experiments were conducted using various elution procedures employing reversed phase and normal phase sorbents. Some of the solvent systems tested for liquid-liquid extraction are methanol-water, ethanol-water, methanol-acetonitrile, ethanol-acetonitrile, chloroform-water, chloroform-acetonitrile, dimethylsulfoxide-water and dimethylsulfoxide-acetonitrile. Following these trials, which demonstrated higher recovery values and increased efficiency from LLE, the following procedure was chosen to be used. These conditions given below are the final liquid-liquid extraction conditions and are used to extract GA and GB from pharmaceutical formulations at the highest yield.

About 0.5 g of pharmaceutical samples was weighed, and 1.5 mL of an ethanol: acetonitrile (1:1) mixture was added to the Eppendorf tube. After 30 seconds of vortexing, the mixture went through a centrifuge for 5 minutes at 4000 rpm. Using 0.45-μm polyethersulfone filters, 800 μL of the supernatant was filtered, and the liquid was then transferred to a 1.5 mL HPLC vial. For every duplicate, 30 μL of the liquid from the vial was administered. The nominal contents of pharmaceutical products were calculated using the regression equations derived from the calibration graphs.

3. Results and discussion

3.1 Method development

The best chromatographic conditions were found by conducting exploratory tests using the HPLC technique. Different temperatures were used to examine different kinds of columns. The best resolution, with more symmetrical and sharper peaks, was obtained using a C18 column that had the following specifications: 250 mm in length, 4.6 mm in diameter and 5 μm particle size, all maintained at 30°C. At varied flow rates, many mobile phases—including both aqueous and acidic solutions—were examined. It was found that the acidic mobile phase produced positive outcomes. As a result, the acidic component was chosen to be a monobasic potassium phosphate solution containing 1 mL/L triethylamine. The organic modifier of choice was acetonitrile. Acetonitrile-monobasic potassium phosphate (pH: 4.4) with a ratio of 90:10 (v/v) at a flow rate of 1.3 mL/min was found to be the ideal mobile phase composition, producing a high-resolution value. We chose two wavelengths, 254 and 280 nm, for quantification. The cosmetic raw material retention durations under these conditions were determined to be 6.10 ± 0.01 for GA and 8.62 ± 0.01 for GB. The ideal conditions for analysis were determined by measuring peak areas and resolution values. The chromatograms in **Figure 4(a–c)** are representative examples. In the previous study, the retention time for GA and GB was 32 and 51 minutes, respectively. This highlights our method as a quality parameter [23].

3.2 Method validation

The International Conference on Harmonization's (ICH) requirements were followed in the validation of the approach [26, 27].

3.2.1 Linearity range and calibration curve

For GA and GB, the linearity ranges for the analysis were set at 0.01–30 μg/mL and 0.05–20 μg/mL, respectively. The following regression equations were found for the calibration curves:

For GA: $y = 104{,}814x - 18{,}554$ (with an r^2 value of 0.9992)

For GB: $y = 51{,}060x - 3200$ (with an r^2 value of 0.9995)

Using the formulae LOD or LOQ = k * SDa/b—where k = 3 for LOD and 10 for LOQ—the values of the limits of detection (LOD) and quantification (LOQ) were calculated. Sda stands for the standard deviation of the intercept, while b is the slope. The LOD values in this case were found to be 0.016 μg/mL for GB and 0.003 μg/mL for GA. Similarly, it was found that the LOQ values for GA and GB were 0.01 and

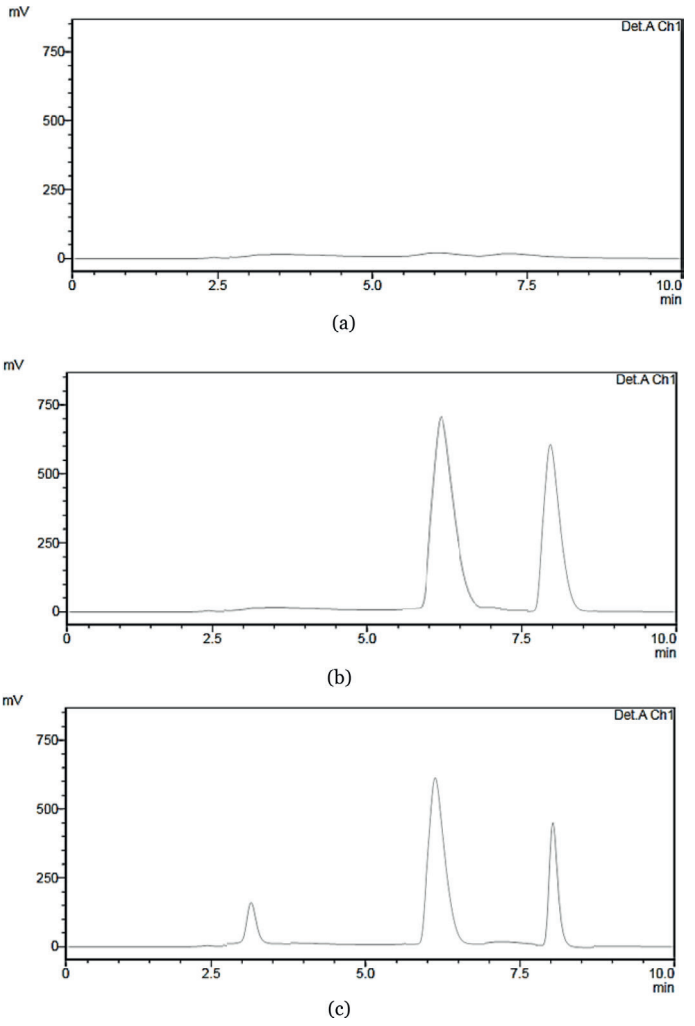


Figure 4.
a: Blank (aqueous medium), b: standard solution (10 µg/mL standard GA and GB solutions), c: real sample (nasal spray).

0.05 µg/mL, respectively. **Table 1** contains further analytical parameters. Although the previous study used a mass spectrometer detector, the LOD value was 0.95 µg/mL, while in our study it was found to be 0.003 µg/mL for GA. This shows that our method is more sensitive [25].

3.2.2 Precision

Three concentration levels (low, medium and high) were used to assess the method's precision in terms of both hourly and daily precisions. Both GA and GB underwent these analyses on the same day as well as seven distinct days, with five runs of each test. For hourly precision, the relative standard deviation (RSD) values varied from 0.25 to 1.57%, and for daily precision, they ranged from 1.30 to 2.85%. These outcomes show how highly repeatable the procedure is.

Parameter	GA	GB
Linearity range* (μg/mL)	0.01–30	0.05–20
Regression equation	y = 104,814x – 18,554	y = 51,060x – 3200
Slope ± SD	104,814 ± 80	51,060 ± 55
Intercept±SD	18,554 ± 35	3200 ± 22
Correlation coefficient, r^2	0.9992	0.9995
LOD (μg/mL)	0.003	0.016
LOQ (μg/mL)	0.01	0.05

**n = 5 correspond to replicate analysis for each level.*

Table 1.
Results of analytical parameters for the proposed method.

3.2.3 Recovery

To ascertain the precision of the technique for quantifying GA and GB, recovery experiments were conducted. The following equation was employed for the recovery trials, and the method's recovery was verified at three different concentrations by spiking the standards to the pharmaceutical formulations listed in **Table 2**.

$$\text{Recovery\%} = \left(\left(\text{Ct} - \text{Cu}\right)\right) / \text{Ca}) \times 100 \quad (1)$$

In the formula, the percentage of the analyte that has been purified is denoted by Ca, the percentage of the analyte that is contained in the formula by Cu, and the total concentration of the analyte that has been found by Ct. The recovery study's results and the industrial pharmaceutical formulation specimen kinds' analysis are presented in **Table 2**. A quantitative range of 97.00–99.01% was seen in the average percent recoveries obtained from the approach. The recoveries in these data are extremely near to 100%, indicating a high degree of accuracy in the approach and a good level of dependability for figuring out the analyte concentration in pharmaceutical

	Existing concentration (μg/mL)	Added concentration (μg/mL)	Found concentration* (mg/mL) (mean ± S.D)	Recovery (%)	RDS of intraday variation	RDS of interday variation
		0.01	1.00 ± 0.02	99.01	0.25	1.30
GA	1	1	1.94 ± 0.04	97.00	1.57	2.85
		30	30.44 ± 0.03	98.19	1.44	2.13
		0.05	1.03 ± 0.02	98.09	1.35	2.06
GB	1	1	1.96 ± 0.03	98.00	1.51	2.57
		20	20.73 ± 0.01	98.71	1.12	2.40

**n = 5 correspond to replicate analysis for each level.*

Table 2.
Results of recovery studies by standard addition method.

formulations. In the previous study, the RSD values were considerably higher than our study, indicating that the reproducibility of the method was low. In addition, all our recovery values were found to be above 95% [24].

3.2.4 Stability

Under a variety of storage settings, such as room temperature in the dark, autosampler conditions for 48 hours and refrigeration at 4°C for a month, the stability of the working standard solutions of the pharmaceutical raw materials was evaluated. The samples continued to be stable in these circumstances, according to the stability study results. In particular, it was discovered that the samples were stable after 48 hours of room temperature storage and the same amount of time under autosampler settings. Additionally, the samples' stability was unaffected by a month of chilling at 4°C. Both GA and GB were shown to be stable under all evaluated storage settings, indicating that sample degradation during storage need not be a worry for the method's ability to accurately assess these pharmaceutical raw materials.

3.2.5 Robustness

By altering important variables such as the flow rate, temperature of the column oven, and the ratios of acetonitrile and the acidic solution in the mobile phase, the robustness of the procedure was assessed. Two distinct ratios—85:15 and 95:5—were substituted for the original 90:10 (acetonitrile-monobasic potassium phosphate solution) composition of the mobile phase. The flow rate was changed from 1.3 to 1.2 and 1.4 mL/min, and the column temperatures were changed from the starting 30–35 and 25°C. Notably, these adjustments to the method's parameters had no discernible effect on the peak regions. **Table 3** shows low relative standard deviation (RSD) values, which shows how reliable the approach is. This implies that the approach may yield consistent and dependable findings even in the presence of small fluctuations in these parameters, which is a crucial feature for its practical usefulness.

3.3 Analysis of real samples

The concentrations of GA and GB in pharmaceutical goods containing *Glycyrrhiza glabra* L. were obtained following the technique and validation experiments and are presented in **Table 4**. The percentage RSD values were quite low in comparison with

Condition	Value	Recovery (%)		RSD (%)	
		GA	GB	GA	GB
Flow rate mL/min	1.2	98.53	99.88	0.24	0.17
	1.4	99.32	99.19	0.29	0.27
Mobile phase composition (ACN:aqueous phase)	85:15	98.96	98.89	1.15	1.21
	95:5	97.15	97.80	1.68	1.61
Column temperature	25	99.16	99.86	0.22	0.16
	35	99.64	99.75	0.31	0.28

For each concentration n = 5.

Table 3.
Results from robustness experiments.

Cosmetic product	Recovery	%RSD	GA (µg/mL)	GB (µg/mL)
Body spray	% 97.8	0.84	24.18	17.98
Cream	% 96.6	1.13	22.97	16.41
Lotion	% 96.4	1.17	22.12	16.04
Ointment	% 95.1	1.65	20.83	14.92
Lozenge	% 95.6	1.54	21.68	15.78
Nasal spray	% 98.1	0.67	26.33	18.44
Nasal ointment	% 97.4	0.91	23.44	17.21
Ear drops	% 92.6	2.24	17.46	13.07
Soap	% 94.4	1.92	19.43	14.01
Shampoo	%93.6	2.10	18.74	13.72

For each concentration n = 5.

Table 4.
Pharmaceutical raw material concentrations and method reproducibility.

our previous work [28]. %RSD values ranged from 0.67 to 2.24. While the amount of GA varied between 26.33 and 18.74 µg/mL, the amount of GB varied between 18.44 and 13.72 µg/mL.

4. Conclusion

We devised a technique in this work to identify commonly used pharmaceutical formulations of GA and GB. GA and GB are utilized in a wide variety of pharmaceutical preparations because they have several medicinal and cosmetic benefits (as indicated by the text's references). The majority of GA and GB are present in *Glycyrrhiza glabra* L. sp. pharmaceutical drugs containing GA and GB are often introduced as preparations made of extract from *Glycyrrhiza glabra* L.

GA and GB are discovered in combination because of their comparable effects. In this regard, our goal was to create a novel HPLC technique that would allow us to determine the concentrations of GA and GB in 10 distinct pharmaceutical types, including nasal spray, shampoo, cream and so on. Our approach also saves costs, is incredibly easy to use and is quite quick. The validation experiments demonstrated the reasonable sensitivity, selectivity, precision and accuracy of this approach. The literature has a few techniques for determining GA and GB, but none of these methods allow analysis of products with such different types of pharmaceutical formulations. Current methods focus on plant extracts, dietary supplements and a single pharmaceutical formulation. In addition to the expensive detector requirements and costs of existing methods, the long retention times constitute a shortcoming. Simultaneous analysis of the maximum absorption of GA and GB at different wavelengths was carried out using the most economical diode array detector (DAD). The method we developed is superior to all methods available in the literature in terms of all validation parameters. The most important of these are retention times, detection limits, sensitivity, selectivity, RSD values and robustness. Retention times of 6.10 minutes for GA and 8.62 minutes for GB were not achieved even by ultra-high performance liquid chromatography. The LOD and LOQ values obtained using the DAD detector could

not be obtained even using the MS detector. Since pharmaceutical formulations are different and unique for each product, there is no cost-effective separation method that removes active ingredients from their environment with such a simple and single extraction method. It also stands out as an environmentally friendly method due to the low amount of solvent used. Moreover, there is no simple extraction method to extract the active ingredients from pharmaceutical products with such different formulations. The approach is anticipated to be useful for pharmaceutical product standardization, quality control and routine analysis, including GA and GB.

Conflict of interest

The author declares no conflict of interest.

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