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Simultaneous Determination of Baicalin and Glycyrrhizin in Eul-Ja-Tang by HPLC/DAD

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Abstract – A high performance liquid chromatographic (HPLC) method for the simultaneous determination of marker constituents, baicalin and glycyrrhizin was established for the quality control of traditional herbal medicinal preparation, Eul-Ja-Tang (EJT). Separation and quantification were successfully achieved with a Waters XTerra RP18 column (5 μm, 4.6 mm I.D. × 150 mm) by gradient elution of a mixture of acetonitrile and water containing 0.03% phosphoric acid (pH 2.03) at a flow rate of 1.0 mL/min. The diode-array UV/VIS detector (DAD) was used for the detection and the wavelength for quantification was set at 250 nm. The presence of baicalin and glycyrrhizin in this decoction was ascertained by retention time, spiking with each authentic standard and UV spectrum. Both baicalin and glycyrrhizin showed good linearity ($r^2 > 0.999$) in a relatively wide concentration ranges. The R.S.D. for intra-day and inter-day precision was less than 5% and the limits of detection (LOD) were about 30 ng. The mean recovery of each compound was 99.5 - 101.2% with R.S.D. values less than 4.0%. This method was successfully applied to the determination of contents of baicalin and glycyrrhizin in three commercial products of EJT, which resulted in the difference in the contents of these compounds. These results suggest that the developed HPLC method is simple, effective and could be readily utilized as a quality control method for commercial EJT products.

Keywords – Eul-Ja-Tang (EJT), HPLC, quantification, validation

Introduction

Traditional herbal medicinal preparations are mostly used in combination of many herbs. Multiple constituents from each herb are known to be responsible for their therapeutic effects (Xue and Roy, 2003), however, the quality of each herb has been affected by many factors such as cultivation environment and manufacturing process (Wang *et al*., 2002; Bennett *et al*., 2004; Lund and Bohlmann, 2006; Antonnen *et al*., 2006). In addition, even though each herb has been mixed in the same ratio, different preparation procedure such as cutting size of herbs, temperature, time, pressure for extraction may affect the amounts of various constituents in the decoction. In other words, all these factors can affect the therapeutic effects and/or safety of traditional medicinal preparation. Therefore, the need for quality assessment of major active components in traditional herbal medicinal preparation has been increased. As such, numerous studies related to quality control have been carried out, mainly by the determination of major and/or active constituents (Sheng *et al*., 2005; Zhang and Cheng, 2006; Huang *et al*., 2006; Li *et al*., 2006).

Eul-Ja-Tang (EJT) is combinational herbal decoction that consists of *Angelica gigas, Scutellaria baicalensis*, *Cimicifuga heracleifolia*, *Glycyrrhiza glabra*, *Bupleurum falcatum*, and *Rheum palmatum*. EJT has been used in traditional medicine to relieve the symptoms of digestive diseases. In addition, EJT has been commercially produced as granules by several medicinal manufactures in Korea. Thus, to ensure the efficacy and safety, a suitable assay method for quality control has been required.

The analytical methods for baicalin in *S. baicalensis* extract and glycyrrhizin in *G. glabra* have been developed respectively in Korean Pharmacopoeia. However, the detection of each compound in EJT has been interfered by the constituents from other plant in EJT. In addition, there

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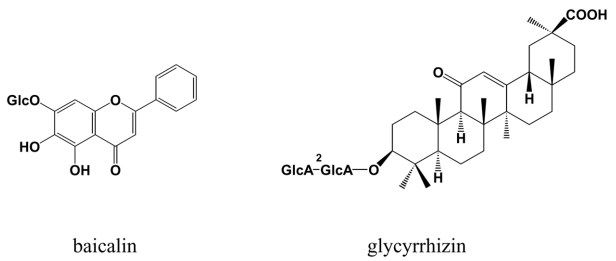

baicalin glycyrrhizin

Fig. 1. Structures of marker constituents of EJT.

have been no reports about the simultaneous quantitative determination of baicalin and glycyrrhizin in EJT.

In the present study, the simultaneous quantitative analytical method for marker constituents of EJT, baicalin from *S. baicalensis* and glycyrrhizin from *G. glabra* (Fig. 1), was developed using HPLC/DAD and validated. In addition, this method was tested using three commercial EJT products.

Experimental

Materials – The standard of marker compounds, baicalin and glycyrrhizin, were purchased from Wako (Osaka, Japan). All of the plants were purchased from Kyungdong traditional herbal market (Seoul, Korea) and were authenticated by Prof. Jong Hee Park in the College of Pharmacy, Pusan National University. Voucher specimens have been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

The commercial EJT products from medicinal companies were purchased from local providers. HPLC grade solvents (acetonitrile, water and methanol) and reagents were obtained from BDH chemicals (Poole, UK). Phosphoric acid (analytical grade) was purchased from Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

Chromatographic conditions – The HPLC system was consisted of a chromatographic pump (P680, Dionex, Germany), an injector (7725i, Rheodyne, USA) equipped with Photo Diode Array (UVD 340U, Dionex, Germany). The output signal of the detector was recorded using a Dionex Chromelon™ Chromatography Data System. Chromatographic separation was achieved on a Waters XTerra™ RP18 (5 μm, 4.6 mm I.D. × 150 mm) by gradient elution of a mixture of acetonitrile and water containing 0.03% phosphoric acid (pH 2.03) at a flow rate of 1.0 ml/min. The diode-array UV/VIS detector (DAD) was used for the detection and the wavelength for quantification was set at 250 nm.

Preparation of standard solution – Stock standard solution of marker compounds was prepared in methanol at a concentration of 1 mg/mL, respectively. The appropriate amount of every standard solution was mixed and diluted as indicated.

Sample preparation for HPLC – For the preparation of EJT sample for HPLC experiment, 6.0 g of *A. gigas*, 3.0 g of *S. baicalensis*, 1.0 g of *C. heracleifolia*, 2 g of *G. glabra*, 5.0 g of *B. falcatum*, 0.5 g of *R. palmatum* were weighed accurately and mixed. Ten times of water (90 ml) was added to mixed herbs for EJT and refluxed for 2 h at 90 °C. The extract was filtered and evaporated in vacuo, and then suspended to 100 ml of 50% methanol. For the preparation of HPLC samples of commercial EJT products, dried commercial EJT granule samples were weighed accurately and extracted with 90% methanol for 1 h at 90 °C, using a reflux. The extraction was repeated three times. All the extract was filtered and evaporated in vacuo, and then suspended to 10 ml of 50% methanol. This sample solution was filtered through 0.45 μm membrane filter (Millipore, Nylon, 170 mm) and analyzed with HPLC.

Results and Discussion

Chromatographic conditions – For the simultaneous determination of marker components of EJT, baicalin and glycyrrhizin, the chromatographic condition was first investigated. Various mixtures of water and acetonitrile in combination with phosphoric acid were tested as a mobile phase. The wavelength for detection was tested at 210, 230, 250 and 280 nm and set at 250 nm, where the marker compounds showed the maximum absorption as measured by a DAD detector. The presence of baicalin and glycyrrhizin in this decoction was verified by comparing each retention time and UV spectrum with those of each standard compound, and spiking with authentic standards. As a result, the optimal gradient mobile phase consisting of acetonitrile-water with 0.03% phosphoric acid was subsequently employed for the analysis of EJT (Table 1), which led to good resolution and satisfactory peak shape at 250 nm (Fig. 2).

Validation of the HPLC Methods – Specificity was determined by the calculation of peak purity facilitated by the photodiode array detector (PDA) and the corresponding computer software. The absorption spectrum of a single component remained little variable at each time point in one peak, which supported specificity of each peak (Fig 2). Our results clearly showed the specificity of each peak

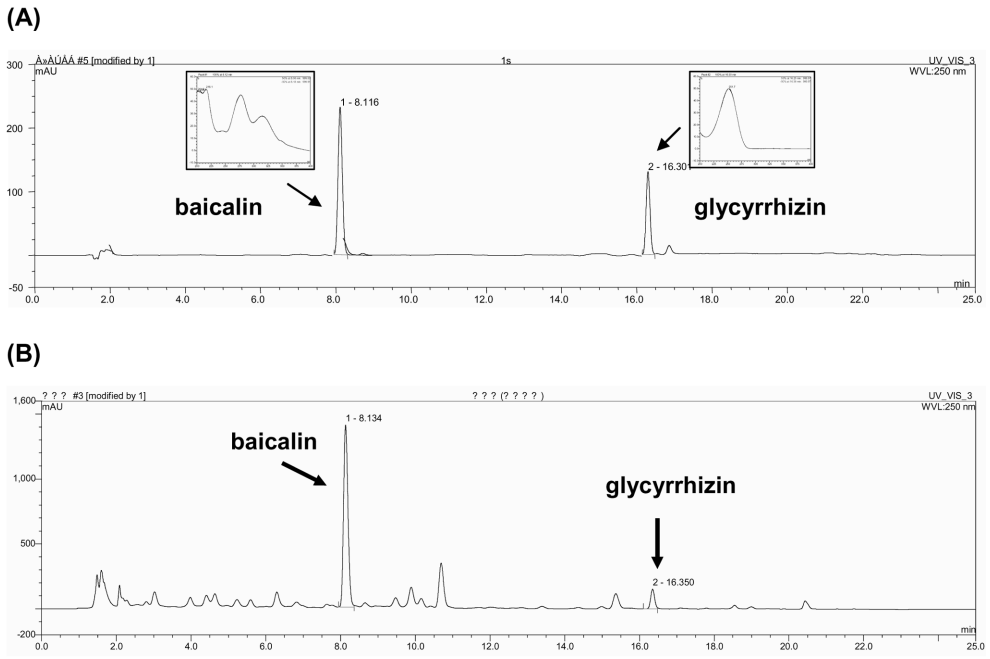

Fig. 2. HPLC chromatogram of standard mixtures (A) and EJT (B).

Table 1. Solvent gradient conditions for HPLC-DAD

Final time (min)	Solvent A[a]	Solvent B[b]	Flow rate (ml/min)
0	20	80	1.0
13	40	60	1.0
20	75	25	1.0
23	20	80	1.0
25	20	80	1.0

Solvent A; Acetonitrile,
Solvent B; 0.03% phosphoric acid (pH 2.03)

for baicalin and glycyrrhizin, respectively.

The linearity of baicalin and glycyrrhizin was calculated by seven concentrations of each compound. The regression equation and correlation coefficients (r^2) were listed in Table 2 and high correlation coefficient values ($r^2 > 0.999$) showed good linearity in relatively wide range of concentration.

Limit of detection (LOD) was measured based on the method recommended by ICH (LOD = 3.3 δ/*S*, δ = standard deviation of the response, *S* = slope of the calibration curve). LOD of baicalin and glycyrrhizin were 37.6 and 30.7 ng, respectively, which showed a high sensitivity at this chromatographic condition.

The precision test was carried out by the intra-day and inter-day variability for these constituents. The intra-day variability was assayed at three concentrations on the same day and inter-day variability was assayed at six concentrations on three sequential days (1, 3, 5 days). As listed in Table 3, the R.S.D. of intra-day and inter-day variability was less than 5.0%, which demonstrated good precision of this method.

The recovery test was determined by the method of standard addition. As listed in Table 4, the mean recovery of each compound was 99.5 - 101.2% with R.S.D. values less than 4.0% ($n = 3$).

Determination of marker compounds in commercial EJT products – The established method has been applied to the determination of marker constituents in commercial EJT granules. Three EJT products by different medicinal

Table 2. Linear ranges, limit of detection (LOD) and characteristic parameters of calibration curves of marker constituents of EJT

Compound	Linear range (μg/mL)	Linear regression equation[a] $y = ax + b$		Correlation coefficient (r^2)	LOD (ng)
		Slope (a)	Intercept (b)		
baicalin	2.0 - 400	7.7973	–0.1123	0.9994	37.6
glycyrrhizin	1.0 - 200	7.7957	0.0060	0.9995	30.7

[a]y = peak area, x = concentration (μg/mL)

Table 3. Analytical results of intra-day and inter-day variability

Compound	Amount (μg)	Intra-day		Inter-day	
		Detected (μg)	R.S.D. (%)	Detected (μg)	R.S.D. (%)
baicalin	2.00	2.02	2.92	2.07	2.28
	0.80	0.85	3.77	0.76	4.66
	0.20	0.21	3.74	0.19	3.46
glycyrrhizin	1.00	1.00	2.64	1.04	2.88
	0.40	0.41	2.45	0.39	4.92
	0.10	0.10	1.96	0.11	3.89

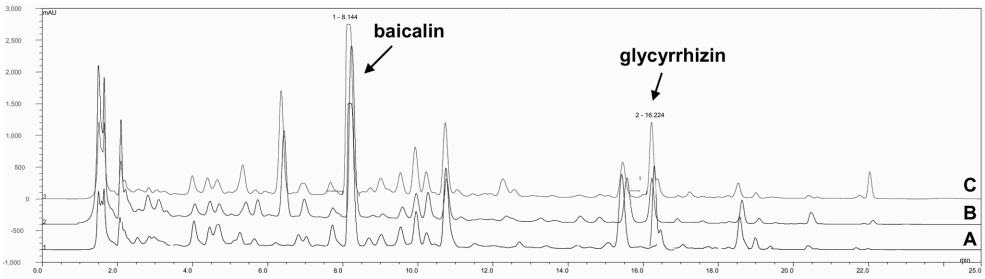

Fig. 3. HPLC chromatogram of three commercial EJT products from different companies, A, B and C.

Table 4. Accuracy for the assay of marker constituents of EJT

Compound	Spiked amount (μg)	Measured amount (μg)	Accuracy (%)	R.S.D. (%)
baicalin	11.76	11.84	100.72	1.20
	9.48	9.55	99.73	0.96
	7.97	8.07	101.26	0.09
glycyrrhizin	1.06	1.17	101.24	1.68
	0.85	0.86	100.56	1.59
	0.71	0.71	99.51	3.77

companies were used for the determination of each compound. As shown in Fig. 3, chromatographic separation of baicalin and glycyrrhizin in each product was well achieved by using the developed method. Therefore, this established method can be readily used for the determination of each compound in EJT products. Moreover, it will save the cost and time for quality control.

Conclusion

In this paper, HPLC method for simultaneous determination of baicalin and glycyrrhizin in EJT has been developed and validated. The method fulfilled all the requirements to be identified as a reliable and feasible method showing good specificity, precision, linearity and accuracy data. In addition, the present study demonstrated that the content of baicalin and glycyrrhizin in three commercial EJT was successfully measured using this method. Therefore, this established method is useful for the quality control of EJT by simultaneous quantitative analysis of these constituents.

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