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Short communication

Determination of 18α-glycyrrhizin and 18β-glycyrrhizin in dog plasma by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method has been developed for the separation and determination of 18α-glycyrrhizin (α-GZ) and 18β-glycyrrhizin (β-GZ) in dog plasma. The two compounds were separated on a reversed-phase column and detected by UV absorption at 254 nm. The mobile phase was a mixture of water–methanol–60% perchloric acid (45:55:0.5, v/v) and was adjusted to pH 8.0 with 25% ammonia solution. Indomethacin was added to the plasma as an internal standard. Methanol was selected for the extraction of both the compounds and internal standard. α-GZ and β-GZ could be precisely determined in concentration of 1 mg/ml in a 0.1 ml sample. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Glycyrrhizin is a saponin, a major effective component of licorice root, having anti-inflammatory, anti-ulcerous and anti-allergic effects. It has recently received more attention as a potential therapeutic agent for several virus diseases including chronic hepatitis and AIDS [1–3]. Hirabayashi et al. have reported the inhibitory effect of glycyrrhizin on the replication of HIV-1 [3]. Commercial glycyrrhizin products generally consist of small amounts of 18α-glycyrrhizin (α-GZ) and larger proportions of 18β-glycyrrhizin (β-GZ) (Fig. 1). However, these stereoisomers cannot be separately determined by the existing high-performance liquid chromatography (HPLC) methods for glycyrrhizin [4,5].

In this study, we have developed a selective HPLC method for the simultaneous analysis of α-GZ and β-GZ in biological samples. This report describes their separation and determination in dog plasma by HPLC with a reversed-phase column using UV detection.

2. Experimental

2.1. Materials

α-GZ and β-GZ were gift from Amato (Fukuchi-

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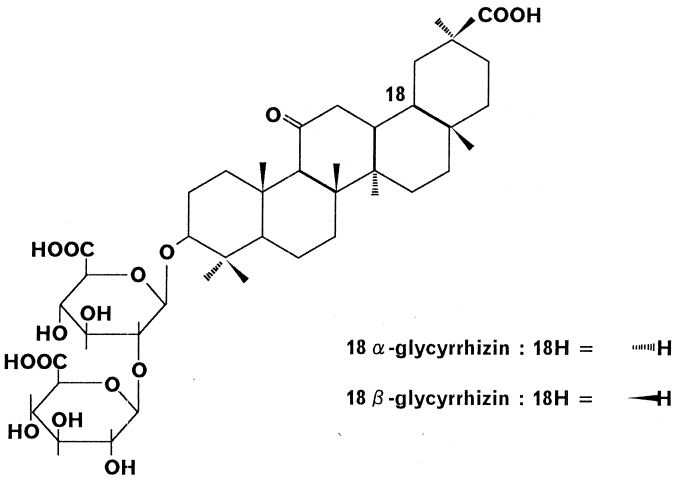

Fig. 1. Structures of 18α-glycyrrhizin (α-GZ) and 18β-glycyrrhizin (β-GZ).

yama, Japan) and Maruzen (Onomichi, Japan), respectively. Methanol, 60% perchloric acid, 25% ammonia solution and indomethacin which was used as an internal standard were purchased from Wako (Osaka, Japan). The stock standard solutions of α-GZ or β-GZ were prepared by dissolving 10 mg of each drug in 100 ml of 50% methanol. Indomethacin was dissolved in 50% ethanol at a concentration of 0.01 mg/ml.

2.2. Chromatographic system

The HPLC system consisted of a Hitachi 655A-40 injector, a Hitachi 655A detector, a Hitachi 655-15 chromatographic pump, and a Hitachi 655-71 data processor (Tokyo, Japan). Separation was achieved on a reversed-phase column (Nucleosil ODS, 5 μm particle size, 250×4.6 mm I.D.). The preparation of mobile phase was as follows: water–methanol–60% perchloric acid (45:55:0.5, v/v) were degassed using an aspirator, and then adjusted to pH 8.0 with a 25% ammonia solution. The flow-rate of the mobile phase was 0.8 ml/min, where the column pressure was 120 kg/cm^2. The chromatogram was monitored at a wavelength of 254 nm throughout the experiments. The column oven temperature was set at 50°C.

2.3. Sample preparation

To 0.1 ml of plasma in a 15-ml glass-stoppered centrifuge tube, 0.1 ml of internal standard solution (0.01 mg/ml indomethacin) and 5 ml of methanol were added. The mixture was shaken for 20 min at a speed of 500 rpm (VR-36D, Taitec, Saitama, Japan), and then centrifuged at 1200 *g* for 20 min (05 PR-2, Hitachi). A 4.5-ml aliquot of the supernatant was transferred to another tube, and then evaporated to dryness with a rotary vacuum evaporator at 60°C. The resultant residue was dissolved in 0.3 ml of the mobile phase, and filtered with a Millipore membrane (LCR13-LH, pore size: 0.5 μm, Japan Millipore, Tokyo, Japan). An aliquot (50 μl) of the filtered solution was injected into the HPLC system.

2.4. Preparation of standard curves

Standard curves were run in duplicate at five different concentrations of α-GZ or β-GZ. Plasma standards were prepared by spiking drug-free plasma with known amounts of a stock solution of α-GZ and β-GZ to produce concentrations of 1, 5, 10, 50 and 100 μg/ml. To 0.1 ml of each prepared standard was added 0.1 ml of drug-free dog plasma and 0.1 ml of the internal standard solution. The standards were then assayed in the described manner. Calibration

curves for α-GZ and β-GZ were generated by plotting the peak-area ratio (analyte/internal standard) versus known concentrations and were fitted using an unweighted linear least-squares regression analysis.

2.5. Recovery

Drug-free plasma (100 μl) was spiked with five different amounts of α-GZ or β-GZ. The samples were pretreated using the method described above. The values of peak-areas measured for α-GZ and β-GZ in the extracted plasma samples over the concentration range of 1–100 μg/ml were compared to the peak-area values for unextracted samples. The recovery was calculated as the peak-area ratio between α-GZ or β-GZ content in the extracted and unextracted samples, multiplied by 100.

3. Results and discussion

3.1. Optimization of HPLC conditions

To optimize the HPLC conditions for the separation of α-GZ and β-GZ, the effects of the pH and mobile phase composition were investigated. The adjustment of pH was performed by varying the proportions of 60% perchloric acid and 25% ammonia solution to achieve a pH range of 2.5–8.0. The mobile phase compositions were prepared with appropriate ratios of either water and methanol or water and acetonitrile. The temperature of column oven was set at 50°C in order to decrease the column pressure. It was shown that separation of the two stereoisomers, α-GZ and β-GZ, was improved with an increase of the pH of the mobile phase. This result suggests that pH is an important factor in the separation. In addition, the separation of the two stereoisomers using water-methanol as a mobile phase was better than that achieved with water-acetonitrile. Furthermore, when a mixture of water and methanol was used as a mobile phase, the sensitivity of detection indicated by peak-area was higher than that obtained with water and acetonitrile. When the pH value was increased to 8.0 by the addition of ammonia solution to the mixture of water–methanol–60% perchloric acid (45:55:0.5, v/v), two peaks due to α-GZ and β-GZ were well resolved with retention times of 11.0 min for α-GZ and 11.9 min for β-GZ, and the chromatographic separation of the two stereoisomers was improved.

3.2. Determination of α-GZ and β-GZ in dog plasma

Fig. 2 shows the chromatograms of blank plasma, α-GZ, β-GZ and blank plasma spiked with α-GZ, β-GZ and internal standard. Blank plasma did not show any interfering peaks in the analytical region (10–18 min) of the chromatograms (Fig. 2A). Although peaks reflecting impurities were observed in chromatograms of standard samples, these peaks did not coincide with retention times of the initial standard (Fig. 2B and C). As shown in Fig. 2D, the isomers and internal standard were detected in dog

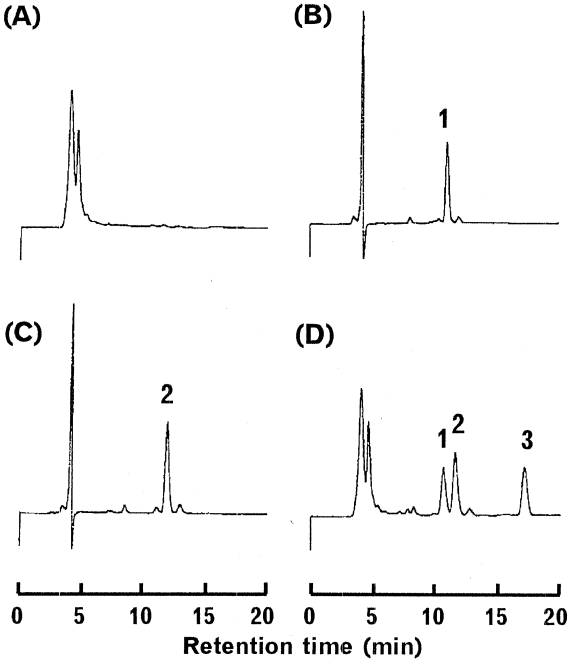

Fig. 2. Chromatograms of α-GZ and β-GZ. (A) Blank plasma; (B) α-GZ in plasma-free solution; (C) β-GZ in plasma-free solution; (D) blank plasma spiked with α-GZ, β-GZ and indomethacin (internal standard). Peaks: 1=α-GZ; 2=β-GZ; 3=indomethacin.

Table 1
Recovery studies of α-GZ and β-GZ in dog plasma[a]

Compound	Spiked concentration (μg/ml)	Concentration retrieved (μg/ml)	Recovery (%)	SD (%)
α-GZ	1	0.98	98.1	
	5	4.87	97.4	
	10	9.90	99.0	2.96
	50	49.0	97.9	
	100	104.6	104.6	
β-GZ	1	0.91	90.8	
	5	4.91	98.2	
	10	9.64	96.4	6.17
	50	50.5	101.0	
	100	107.6	107.6	

[a] The concentration retrieved and recovery values were the means of two experiments. The listed SD was the SD of the mean of five recovery values.

plasma. There were no peaks of impurity included for internal standard (data not shown).

3.3. Linearity

Linearity of the calibration curves of α-GZ and β-GZ was studied. The peak-area ratios of the two standards to the internal standard were found to be linear throughout a range of 1–100 μg/ml ($y=0.0148x+0.0370$, $r=0.9996$ for α-GZ and $y=0.0198x+0.0277$, $r=0.9998$ for β-GZ). The relative standard deviations (RSDs) for the measurement of multiple samples were all less than 7.0%.

3.4. Recovery

The efficiency of recovery was evaluated by analyzing plasma samples containing known amounts of α-GZ, β-GZ and internal standard. The recoveries of α-GZ and β-GZ using this analytical procedure were in the range of 97.4–104.6% and 90.8–107.6%, respectively (Table 1). These values are in good agreement with that reported for glycyrrhizin the literature [4]. The standard deviations (SDs) of the mean of five recovery values were 2.96% for α-GZ and 6.17% for β-GZ.

4. Conclusion

Pharmacological activities of the glycyrrhizin stereoisomers have not been separately investigated so far. A selective HPLC method for the determination of α-GZ and β-GZ in dog plasma was established. The separation of both compounds from plasma was easily achieved by extraction with methanol and by setting the pH of the mobile phase at pH 8.0. Moreover, the temperature of the column oven was kept at 50°C. Recoveries from plasma spiked with α-GZ and β-GZ were over 97% and 90%, respectively. The SD of the mean of recovery values of five concentrations was sufficiently low for both compounds. α-GZ and β-GZ can be isolated and determined quickly with an acceptable recovery from biological samples by the HPLC system. The method is applicable to pharmacokinetic studies of GZ in dogs, and should be useful in pharmacokinetic and pharmacodynamic studies on glycyrrhizin in animals and humans.

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