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Determination of Total Glycyrrhetic Acid in Glycyrrhizae Radix by Second Derivative UV Spectrometry

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Abstract□Second derivative (D2) spectrometry using ion-pair extraction technique was developed for the determination of total glycyrrhetic acid (GA) in Glycyrrhizae Radix. Glycyrrhizin (G) obtained from Glycyrrhizae Radix was hydrolyzed into GA in 2 N-HCl and methanol (1:1) and extracted from aqueous phase in the form of an ion-pair complex with tetrapentylammonium bromide (TPA) as a counter ion. Maximum D2 amplitude (Z value) was obrained when 1000-fold or greater molar ratio of TPA was used at pH 11. Reaction time, temerature and ionic strength did not affect ion-pair formation. Dichloromethane was an effective extraction solvent of the ion-pair complex. The linearity of standard curve of ion-pair GA was obtained in the range of 4-120 μg/ml as GA. Assayed contents of GA in dry powder by D2 UV spectrometry and HPLC method were $5.31 \pm 0.04\%$ and $5.20 \pm 0.008\%$, respectively.

Keywords□Glycyrrhizae Radix, second derivative, UV spectrometry ion-pair complex, glycyrrhetic acid, glycyrrhizin.

Since Collier and Singleton had developed the concept of the second derivative spectrometry in 1956, high order derivative technique (HODT method) have been applied in many analytical fields. Derivative spectra make it possible to determine flat maxima more precisely and to isolate spectrum shoulder and weak signals from unwanted interferences. HODT was available for trace anaysis[1], purity test[2], stability test[3], assay of turbid solutions, suspensions and emulsions, analysis of superimposed spectra of mixtures[4-6] and assay of preparations[7,8].

The first derivative of a Gaussian absorption band ($dA/d\lambda$) has often been used to detect and locate 'hidden' peaks (since it passes through zero at the peak maximum), it transpires that the second, and even higher, derivatives of absorbance or signal intensity ($d^2A/d\lambda^2$, $d^4A/d\lambda^4$) are potentially more useful analytically. The basis of this behavior lies in the observation that, for Gaussian bands, the amplitude, Z_n of the nth derivative is inversely related to the original band-width, W, raised to the nth degree:

$$Z_n \propto W^{-n}$$

so that for 2 coincident bands of equal intensity, the nth derivative amplitude of the sharper band x exceeds that of the broader band y by a factor which increases with derivative order:

$$\frac{Z_{n,x}}{Z_{n,y}} \propto \left(\frac{W_y}{W_x}\right)^n$$

This feature may contribute to an increase in the detection sensitivity in second and fourth derivative spectra. For quantitative analysis in the derivative domain, if it can be assumed that the Beer-Lambert law is obeyed over the concentration range of interest in the zero-order spectrum, then:

$$\frac{d^nA}{d\lambda^n} = \frac{d^nE}{d\lambda^n} \cdot b \cdot c$$

where A = absorbance, E = molar absorptivity ($M^{-1}cm^{-1}$), b = cell path-length (cm) and c = molar concentration (M)[9].

The Glycyrrhizae Radix is one of the most fundamental crude oriental drugs that are extensively used in traditional medicine. G and GA are main components of Glycyrrhizae Radix and their pharmaceutical activities have been widely investigated. GA has been measured by various methods including TLC[10,11], spectrophotometry[12], HPLC[13-19], GC[20], and enzyme immunoassay[21,22]. The purpose of this study is to establish a simple UV spectrophotometric analysis of total GA with the combination of ion-pair exrtraction technique and D2 UV spectrometry. This could provide a simple, economical and convenient determination method of GA for easy quality control

of Glycyrrhizae Radix and its preparations.

EXPERIMENTAL METHODS

Apparatus

Spectral measurement system used was a Perkin-Elmer Lamda 5 UV/VIS double beam spectrophotometer. HPLC system was consisted of a constant flow dual pump and a variable wavelenght UV single beam detector (Pye Unicam 4010). The column was a Partisil (particle size 10 μm silica, 25 cm × 4.6 mm I.D.; Pye Unicam).

Material and reagent

18 β-GA was purchased from Sigma (St. Louis, MO, USA). Tetramethylammonium bromide (TMA), tetrapropylammonium hydroxide (TPrA), tetraethylammonium bromide (TEA), tetrabutylammonium phosphate (TBA), tetrapentylammonium bromide (TPA), and tetraoctylammonium bromide (TOA) were purchased from Aldrich (Milwaukee, WI, USA). Dry power and extract of Glyrrhezae Radix (*Glycyrrhiza uralensis*) were purchased from a market. All chemicals used were of analytical-reagent grade, unless specified otherwise. Deionized, distilled water was used.

Extration and hydrolysis

Dry power of Glyrrhizae Radix (0.01 g) was weighed accurately, placed in 50 m*l* of the solution of 2 N-HCl: methanol (1:1) and refluxed on a heating mantle at 80°C for 90 min. Methanol was evaporated and 2 N-NaOH solution was added to adjust pH 11. This solution was filtered (Millipore, 0.45 μm) and the residue was washed twice with 10 m*l* distilled water. The extract and washings were put into a 100 m*l* measurintg flask and made up to 100 m*l* with distilled water for a sample stock solution. For the standard, 4.07 mg of 18 β-GA was weighed accurately and 50 m*l* of mixture of 2 N-HCl and methanol (1:1) was added. The rest of procedure was same as for the sample.

Extraction of ion-pair complex

10 m*l* of stock solution of GA was transferred into 60 m*l* separating funnel and mixted with the same volume of TPA stock solution (1×10^{-1} M) shaking for a min at room temperature and kept in standing for 15 min. For the extraction of ion-pair complex, 10 m*l* of dichloromethane was added and shaken vigorously for two min and allowed to stand the mixture until the phase separation was completed. The organic layer was transferred into a 50 m*l* volumetric flask and repeated the same procedure twice. Dichloromethane was added up to the volume.

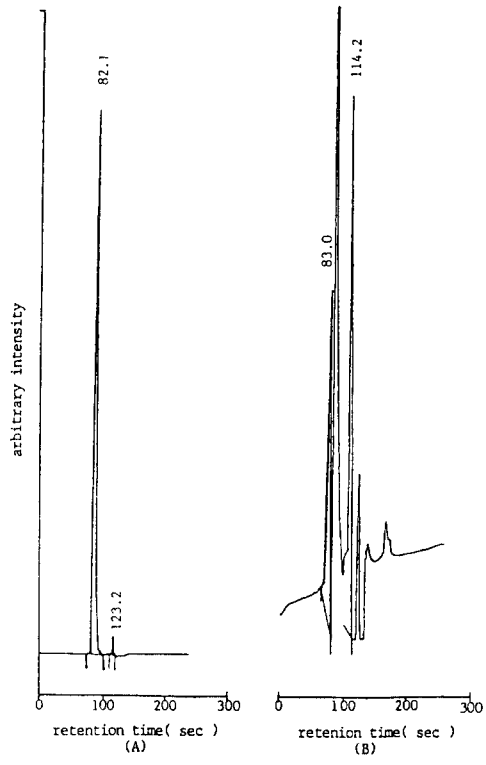

Fig. 1. HPLC chromatograms of glycyrrhetic acid (A) and extract of Glycyrrhizae Radix (B).

D2 UV spectrometry

For the determination of GA in a sample extract, standard addition method was used. 100,200,300 μ*l* of standard stock solution of GA were transferred into the each separating funnel containing 10 m*l* of sample extract. Total ion-pair complex with TPA was extracted with dichloromethane following the procedure described. D2 UV spectra of the samples were recored. The contents of GA in power and extract were calculated in comparision with the D2 amplitudes (Z value at 255 nm).

HPLC determination

Hydrolyzed sample stock solution (10.0 m*l*) was treated with 2 N-HCl to acidify it then extracted with dichloromethane. The organic phase was dryed and redissolved in methanol for the HPLC injection. Mobile phase for HPLC was a mixture of methanol and chloroform (1:1). Flow rate was 3.0 m*l*/min at room temperature. The eluent was monitored at

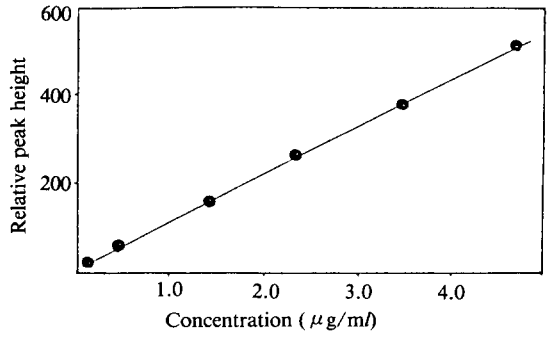

Fig. 2. Calibration curve of glycyrrhetic acid by HPLC method.

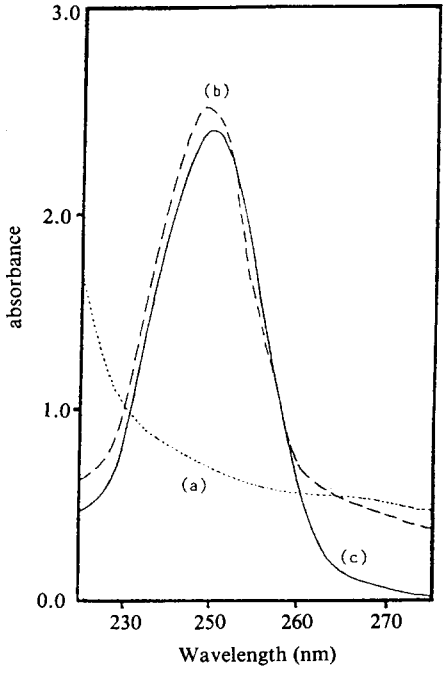

Fig. 3. Zero-order spectra of GA.
Key: a, Extract solution without ion-pairing; b, Sample stock solution, ion-paired with TPA; c, Standard GA stock solution, ion-pair with TPA.

250 nm. Each 10 μl of the pretreated stock solutions of extract and power was injected. The assayed contents of GA in power and extract were calculated in comparison with the peak height (retention time: 82.1 sec) with that of standard GA (Fig. 1). A calibration curve was prepared using GA as a standard solution (Fig. 2).

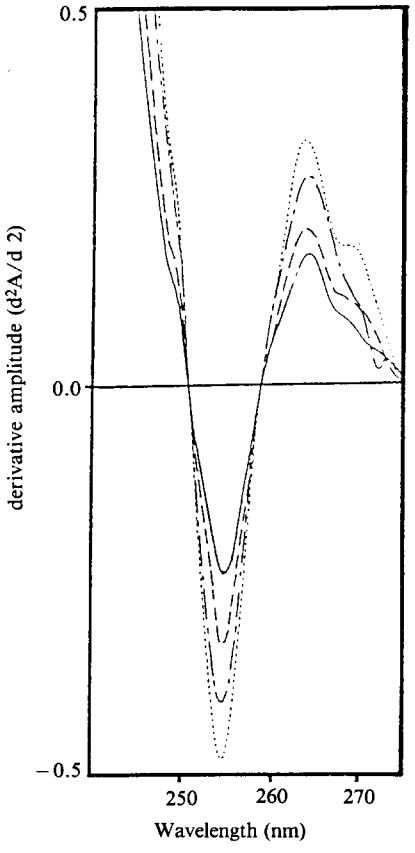

Fig. 4. D2 UV spectra of extract of Glycyrrhizae Radix after adding various volumes of GA standard solution (1×10^{-4}).
Key: ——— , Ion-pair extract of Glycyrrhizae Radix; – – – – , Added 100 μl; —·—·—, Added 200 μl; ·····, Added 300 μl.

RESULT AND DISCUSSION

Zero-order and D2 UV spectra of ion-pair complexes

As shown in Fig. 3, zero-order spectrum of the extract without ion-pairing (a) showed no absorbance maximum. However, the spectrum of ion-pair extract with TPA (b) showed absorbance maximum at 249 nm corresponding to the maximum peak of ion-pair GA complex with TPA (c). Although the spectrum of ion-pair extract showed a peak, it also had a strong backgroud due to some other matrices. The technique of derivative UV spectrophotometry had been proved particulary in eliminating matrix background.

The D2 spectrum of ion-pair GA complex with TPA was given in Fig. 4. Net amplitude (Z value) of GA could be obtained using the derivative ampitude

Table I. D2 amplitude of counter ions on the formation of ion-paired complex

Counter ion	Molecular Weight	Z value
Tetramethylammonium bromide (TMA)	133.9	0.36
Tetraprophylammonium hydroxide (TPrA)	203.37	0.43
Tetraethylammonium bromide (TEA)	210.16	0.39
Tetrabutylammonium bromide (TBA)	339.46	1.29
Tetrapentylammonium bromide (TPA)	378.4	1.51
Tetraoctylammonium bromide (TOA)	546.82	1.63

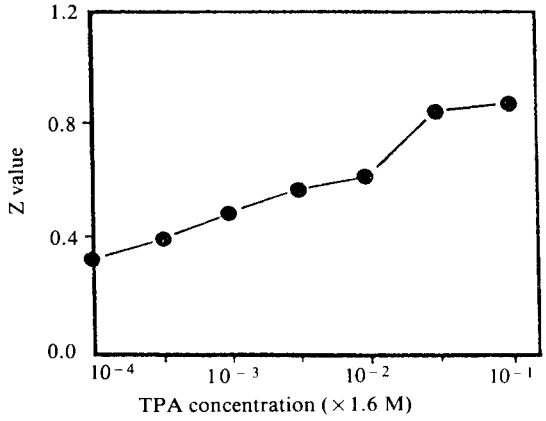

Fig. 5. Effect of TPA concentration on the formation of ion-pair complex with GA from Glycyrrhizae Radix.

unit at 255 nm. The derivative amplitudes were increased proportionally with the concentration of the added standard GA.

Ion-pair effect of counter ions

Equal volumes of TMA, TPrA, TEA, TBA, TPA (1.0×10^{-1} M in distilled water) and TOA (1.0×10^{-1} M in 30% (v/v) methanol) were added to 10 m*l* aliquots of GA standard solution. The effect of various counter ions was illustrated in Table I. High molecular weight of counter ion had high Z value. TBA, TPA, and TOA indicated appreciable Z values. However, TPA was chosen as a counter ion because of water solubility ion-pair formability.

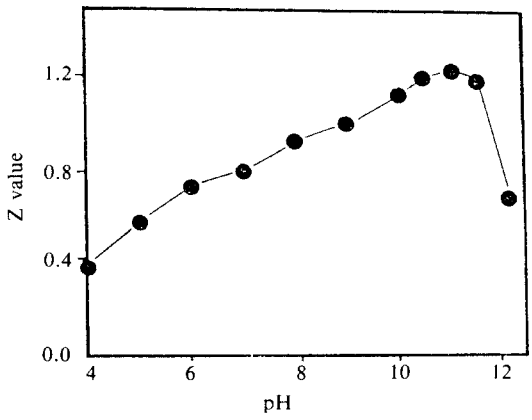

Fig. 6. Influence of pH on the formation of ion-pair complex.

Effect of TPA concentration

To find out the adequate counter ion concentration, 10 m*l* of various concentrations (1.6×10^{-4} M-3.2×10^{-1} M) of TPA was added to 10 m*l* of the extract solution. The Z values were increased with the TPA concentration up to 3.2×10^{-2} M as showed in Fig. 5.

Influece of pH on the formation of ion-pair complex

The pH of stock solution of sample extract was varied from 4 to 12 (Fig. 6) by adding 2 N-HCl and/or 2 N-NaOH. Z values were increased with pH and dramatically dropped at pH 11.5. In the subsequent experiments, the pH of the stock solution was fixed at 11.

Ion-pair effect according to reaction time, temperature and ionic strength

Reaction temperature was varied from 25°C to 65°C (Fig. 7). However, there was no significant difference in Z value. Reaction time and ionic strength also did not affect on Z value (Data not shown). Therefore, subsequent experiment was carried out at room temparture.

Extracting effect of solvents on ion-pair complex

Extraction efficiency of ion-pair complex was tested with various solvents. The result was shown in Table II. Ethylacetate and chloroform were excluded due to poor phase separation. Dichloromethane was chosen as an extraction solvent in this experiment.

Calibration curve by D2 UV spectrometry

Concentration of standard stock solution

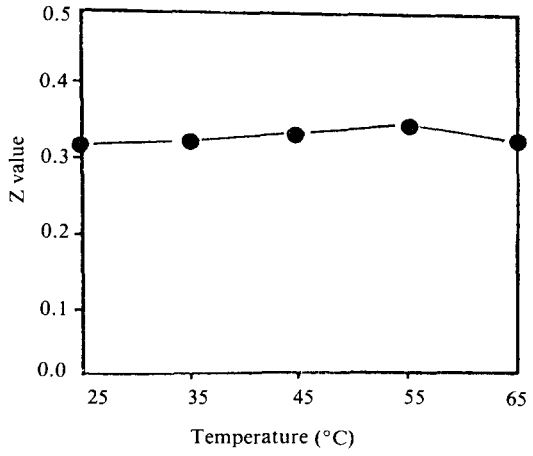

Fig. 7. Infuence of reaction temperature on the formation of ion-pair complex.

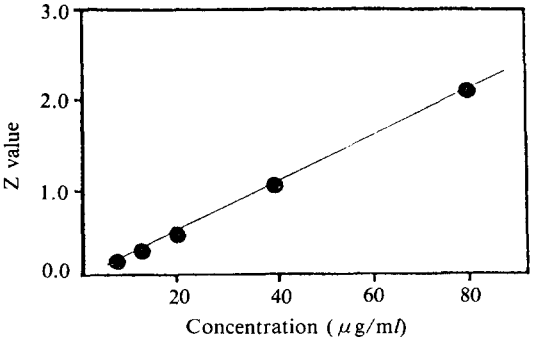

Fig. 8. Calibration curve of ion-pair GA complex by D2 spectrometry.

(1×10^{-3} M) of GA was varied from 1×10^{-5} to 1×10^{-3} M. 10 m*l* aliquot of standard solution was treated following the preceeding assay procedure. A calibration curve was prepared by measuring the Z value at 255 nm of the D2 spectra. Linear regression analysis of Z values versus standard concentrations gave a slope of 0.028, an intercept of −0.088, and a correlation coefficient, r, of 0.9998 (n = 6) as shown in Flg. 8.

Determination of GA by D2 UV spectrometry and HPLC

The assay results of GA in dry power were in Table III and IV. Assayed contents of GA by D2 UV spectrometry and HPLC were 5.31 ± 0.04% and 5.20 ± 0.008% respectively, showing almost the same results. Extract of Glycyrrhizae Radix was assayed by D2 UV spectrometry and the mean content was 11.41 ± 0.11%.

Table II. D2 amplitude on extraction of ion-pair complex

Solvent	Z value
Dichloromethane	2.65
Ether	0.91
Chloroform	2.78
Ethylacetate	2.54

Table III. GA contents assayed by D2 UV spectrometry

Sample	Z value[a]	Contents (%)	
		0.209	5.31
	0.211	5.35	
Dry	0.210	5.33	mean[b] =
Power	0.206	5.26	5.31 ± 0.04
	0.210	5.33	RDS = 0.59%
	0.208	5.30	
	0.552	11.44	
	0.550	11.41	
Crude	0.549	11.34	mean =
Extract	0.543	11.58	11.41 ± 0.11
	0.551	11.43	RDS = 0.76%
	0.557	11.57	

[a]: Based upon triplicated determination of each sample
[b]Confidence limits at 95% level

Table IV. GA contents assayed by HPLC method

Sample	Relative peak height[a]	Contents (%)	
	15.092	5.195	
	15.152	5.200	mean[b] =
Dry	15.064	5.191	5.20 ± 0.008
Power	15.101	5.200	RSD = 0.13%
	15.125	5.211	
	15.106	5.202	

[a]Based upon triplicated determination of each sample
[b]Confidence limits at 95% level

CONCLUSION

This assay method combinded with ion-pair extraction technique and D2 UV spectrometry provided a simple, accurate and convenient mean for the determinaiton of GA in Glycyrrhizae Radix. This method is expected to be applied for the quality control of Glycyrrhizae Radix and its preparations.

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