*Journal of Chromatography*, 347 (1985) 438–442
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 082

## Note

# Analysis of isoflavones in *Puerariae radix* by high-performance liquid chromatography with amperometric detection

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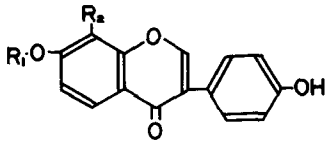
(First received June 3rd, 1985; revised manuscript received August 3rd, 1985)

The *Puerariae radix*, which is a major component of Kakkontou, is used as one of the important herbs in traditional chinese medicine. Isoflavones such as puerarin [8-β-D-glucopyranosyl-7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one], daidzin [7-(β-D-glucopyranosyloxy)-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one], and daidzein [7-hydroxy-3-(4-hydroxyphenol)-4H-1-benzopyran-4-one] have been isolated from the herb[1–3]. Pharmaceutical studies on the biological action of these compounds, *e.g.* antipyretic action[4], papaverin-like action[5,6], cholinergic action[7], the hydrothermic effect, the spasmolitic effect[8], etc. have been reported. Also, in the course of our isolation study on biologically active substances in this herb, we found that daidzin shows anti-inflammatoric action and depresses the bleeding volume[9].

Previously, a high-performance liquid chromatography (HPLC) technique based on a reversed-phase system with UV and/or fluorescence (FL) for detecting isoflavones in *Puerariae radix*[10,11], soybean[12–15] and others[16–18], has been reported. A highly sensitive, analytical method for detecting isoflavones is important for studies on their metabolism. Considering the nature of their phenolic group (see Fig. 1) in electrochemical oxidations, they should be highly sensitive to amperometric detection[19,20]. We found that amperometric detection is very useful for perceiving the three isoflavones in *Puerariae radix*. The proposed method is highly sensitive compared to other methods.




Puerarin: $R_1$=H, $R_2$=glucosyl
Daidzin : $R_1$=glucosyl, $R_2$=H
Daidzein: $R_1$=$R_2$=H

Fig. 1. Structures of isoflavones.

0021-9673/85/$03.30 

## EXPERIMENTAL

The three isoflavones were isolated from commercial *Puerariae radix*. *Puerariae radix* was extracted with methanol and water (1:1) under reflux, and the extract was partitioned with *n*-butanol and water. The butanol fraction was subjected to silica gel column chromatography (74–149 μm, Wako, Osaka, Japan) with a solvent system of chloroform–methanol–water (X:1:0.1, where X = 9–5) to give the following three compounds[1]–3.

(1) Puerarin (recrystallized from acetic acid); m.p. 206–208°C; $C_{21}H_{20}O_9 \cdot H_2O$. IR$\nu_{max}^{KBr}$($cm^{-1}$): 3400, 3250, 1703, 1635, 1618, 1580, 1265. UV$\nu_{max}^{MeOH}$m$\mu$(log ε): 304(3.93), 249(4.40). MS(*m/e*): 395, 362, 293, 256. NMR (in $d_6$-DMSO) δ(ppm): 9.50(1H,br), 8.32(1H,s), 7.93(1H,d,$J$=8.7 Hz), 7.39(2H,d,$J$=8.4 Hz), 6.97(1H,d,$J$=8.7 Hz), 6.79(2H,d,$J$=8.4 Hz), 4.35–5.03(5H,m), 3.90–4.08(2H,m).

(2) Daidzin (recrystallized from methanol); m.p. 247°C (234–236°C)[21]; $C_{21}H_{20}O_9 \cdot H_2O$. IR$\nu_{max}^{KBr}$($cm^{-1}$): 3420, 3270, 3000, 1635, 1443, 1270, 1095. UV$\nu_{max}^{MeOH}$m$\mu$(log ε): 259(4.78), 250(sh)(4.76), 231(sh)(3.67). MS(*m/e*): 416, 254, 225, 137. NMR (in $d_6$-DMSO) δ(ppm): 9.50(1H,bs), 8.34(1H,d,$J$=8.8 Hz), 7.40(2H,d,$J$=8.3 Hz), 8.00–8.32(2H,m), 5.35–5.45(1H,m), 4.83–4.20(5H,m), 4.50–4.63(1H,m).

(3) Daidzein (recrystallized from 50% ethanol solution); m.p. 320–321°C; $C_{15}H_{10}O_4$. IR$\nu_{max}^{KBr}$($cm^{-1}$): 3420, 3270, 3000, 1635, 1445, 1270, 1095. UV$\nu_{max}^{MeOH}$m$\mu$(log ε): 299(sh)(3.95), 248(4.36). MS(*m/e*): 254, 225, 195, 137. NMR (in $d_6$-DMSO) δ(ppm): 9.50(1H,br), 8.20(1H,s), 7.96(1H,d,$J$=8.4 Hz), 7.17(2H,d,$J$=8.2 Hz), 6.60–7.00(4H,m).

The mobile phase for HPLC was prepared by mixing acetonitrile and 0.1 *M* potassium dihydrogen phosphate solution acidified with phosphoric acid to pH 4.0 (15:85).

A Shimadzu HPLC model LC-3A (Shimadzu Seisakusho, Kyoto, Japan) equipped with a column oven (Shimadzu CTO-2A) was used to deliver the mobile phase at a flow-rate of 1.0 ml/min. Two reversed-phase columns, LiChrosorb RP-8 (5 μm, 250 × 4 mm I.D., Merck, Darmstadt, F.R.G.) and IRICA ODS (5 μm, 250 × 4 mm I.D. IRICA-Kogyo, Kyoto, Japan) were used, which were octylsilane and octadecylsilane-bonded columns, respectively. The columns were thermostated with an oven at 50°C.

A IRICA E-502 amperometer operated at a potential setting of +1.00 V *vs.* Ag/AgCl, a Shimadzu SPD-1 spectrophotometer at a wavelength 254 nm, and a Shimadzu RF-510LC spectrofluorometer with excitation at 320 nm and emission at 470 nm, were used in series.

The sample solution was prepared as follows, by Hayakawa's method[11]; *Puerariae radix* (1 g) was extracted with 200, 150 and, finally, 150 ml of methanol under reflux. The combined methanol extracts were concentrated to less than 10 ml by evaporation. The concentrate was treated with a Waters Sep-Pak $C_{18}$ cartridge (Millipore, Bedford, MA, U.S.A.) and filled to 500 ml with methanol. A 1-ml aliquot of this methanol solution was diluted to 50 ml with methanol.

## RESULTS AND DISCUSSION

To determine the optimum applied voltage of the amperometric detector, the peak heights of puerarin, daidzin and daidzein were measured at various potentials in the range from +0.60 to +1.10 V *vs.* Ag/AgCl. Fig. 2 shows that the peak heights of these compounds increased with applied potential, and the curves of hydrodynamic voltammograms of puerarin, daidzin and daidzein were sigmoidal. Taking into consideration the intensity of the dark current and the interference of the component from the herb, the applied potential of the amperometric detector was set at +1.00 V *vs.* Ag/AgCl.

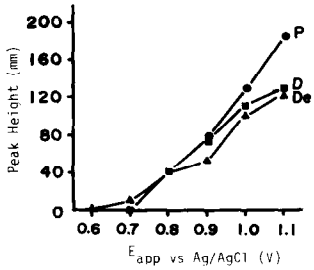



Fig. 2. Hydrodynamic voltammograms of puerarin, daidzin and daidzein: P, puerarin (10 ng); D, daidzin (10 ng); De, daidzein (10 ng). Conditions: column, LiChrosorb RP-8 (5μm, 250 × 4 mm I.D.); mobile phase, 0.1 *M* $KH_2PO_4$ (pH 4.0)–acetonitrile (85:15); flow-rate, 1.0 ml/min; column temperature, 50°C.

In order to determine the effect of pH on the capacity factors of these compounds, the pH was changed in the range 3–8 by using phosphate buffer. The pH was adjusted with 0.1 *M* $KH_2PO_4$ and phosphoric acid. The capacity factors of puerarin and daidzein were constant in the pH range 3–6 but decreased at a pH of more than 7, while that of daidzin remained constant in the studied pH range (Fig. 3). On this basis, acidic phosphate buffer set at pH 4.0 was used as the mobile phase. The effect of the concentration of the phosphate buffer on the peak heights and their capacity factors in the mobile phase was examined. As seen in Fig. 4, the maximum

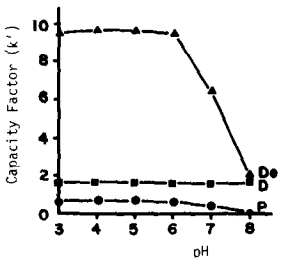



Fig. 3. Effect of pH of phosphate buffer mobile phase on the capacity factor. Conditions: applied voltage, +0.80 V *vs.* Ag/AgCl; others as in Fig. 2.

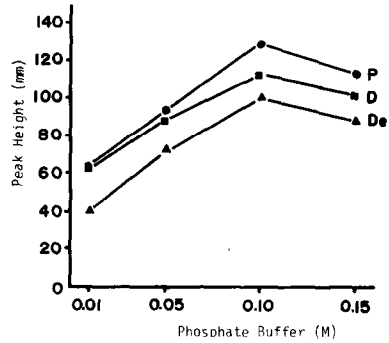
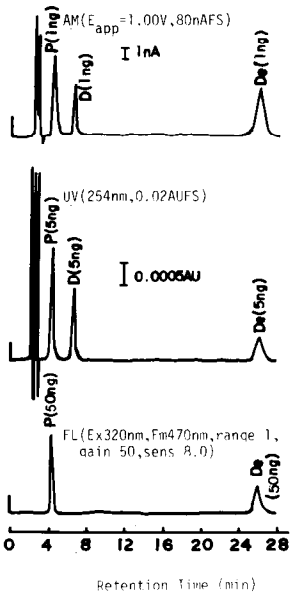



Fig. 4. Effect of concentration of phosphate buffer mobile phase on peak height. Conditions: applied voltage, +1.00 V *vs.* Ag/AgCl; others as in Fig. 2.



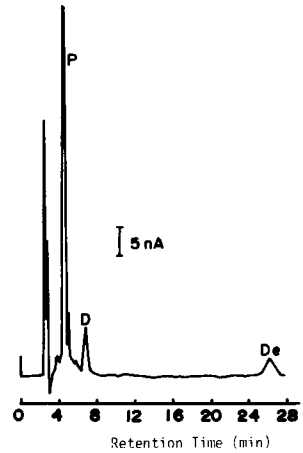




Fig. 5. Comparison of sensitivity with different methods of detection: AM, amperometric; UV, ultraviolet; FL, fluorescence. Conditions: as in Fig. 2.

Fig. 6. Chromatogram of methanol obtained from *Puerariae radix* extract. Conditions: applied voltage, +1.00 V *vs.* Ag/AgCl; range, 160 nA.f.s.

peak heights of these compounds were obtained with 0.1 *M* buffer, and the capacity factors were constant in the examined range.

Under the conditions described above, these compounds were well separated and completely eluted within 28 min in a LiChrosorb RP-8 column and within 35 min in a IRICA ODS column. They were detected at a level of 1.0 ng. As illustrated in Fig. 5, each compound was detected with the highest sensitivity when using amperometric detection as opposed to the other two methods of detection. In a chromatogram of sample solution (Fig. 6), the contents of puerarin, daidzin and daidzein in the extract of *Puerariae radix* were 2.96, 0.32 and 0.13%, respectively (Table I).

In conclusion, the simultaneous analysis of puerarin, daidzin and daidzein in *Puerariae radix* by HPLC was achieved. These compounds were perceived with high sensitivity by using amperometric detection as compared with UV and FL detection. The method described here is applicable to the study of the metabolism of *Puerariae radix*, other *Puerariae* spp. and their components.

TABLE I

DETERMINATION OF PUERARIN, DAIDZIN AND DAIDZEIN IN THE EXTRACT OF *PUERARIAE RADIX*

| *Isoflavone* | *n* | *Mean (%)* | *Standard deviation (%)* | *Coefficient of variation (%)* |
|---|---|---|---|---|
| Puerarin | 3 | 2.96 | 0.079 | 2.67 |
| Daidzin | 3 | 0.32 | 0.009 | 2.81 |
| Daidzein | 3 | 0.13 | 0.005 | 3.85 |

ACKNOWLEDGEMENTS

Thanks are due to Dr. H. Ishida for IR, MS, NMR and UV spectra recording and for criticism of the manuscript, and to O. Miura and K. Yamamoto (Kanebo Ltd.) for instructive discussion.

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