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Journal of Chromatography A, 803 (1998) 179–187

JOURNAL OF CHROMATOGRAPHY A

Separation of nine iridoids by capillary electrophoresis and high-performance liquid chromatography

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Received 7 July 1997; received in revised form 11 November 1997; accepted 28 November 1997

Abstract

A capillary zone electrophoretic (CZE) method and a high-performance liquid chromatographic (HPLC) method were developed for the separation of the nine iridoids, gardenoside, geniposide, geniposidic acid, shanzhiside, loganin, loganic acid, aucubin, harpagoside and catapol. Detection at 210 and 230 nm with a 2,6-di-O-methyl-β-cyclodextrin and sodium borate buffer as carrier or with a linear gradient elution system using acetonitrile and potassium dihydrogenphosphate solution as eluent was found to be the most suitable approach for this separation. The CZE analysis time (32 min) was shorter than that of HPLC (45 min), but the CE method can separate only eight of the nine compounds. The pH, buffer concentration and organic composition of the mobile phase were studied for their effects on the separability of the compounds. © 1998 Elsevier Science B.V.

Keywords: Buffer composition; Mobile phase composition; Iridoids

1. Introduction

Iridoids and their glycosides exist broadly in plants of many families especially the Rubiaceae, Pyrolaceae and Scrophulariaceae. Compounds of this category commonly possess a bitter taste and, in addition, a wide variety of biological activities. According to the literature, the therapeutic effects of many Chinese herbs are attributable to these compounds [1]. The most common method currently used for the analysis of these compounds is high-performance liquid chromatography (HPLC) which is usually limited to the determination of one to three iridoids in specific plants [2–7]. In this study nine compounds (Fig. 1), namely gardenoside, geniposide, geniposidic acid and shanzhiside from *Gardeniae fructus*, loganin and loganic acid from *Corni fructus*, aucubin from *Plantaginis semen*, harpagoside from *Scrophulariae radix* and catalpol from *Rehmanniae radix* [8], were separated successfully by HPLC and capillary electrophoresis (CE). Moreover, the effects of borate concentration and 2,6-di-O-methyl-β-cyclodextrin concentration in CE and the dihydrogenphosphate concentration, pH value and organic modifier composition in HPLC were also investigated.

2. Experimental

2.1. Reagents

Geniposide (GP), aucubin (AU), catalpol (CT) and geniposidic acid (GPA) were purchased from

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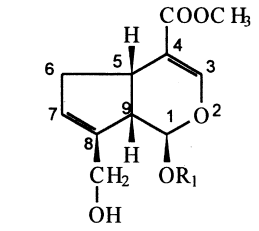
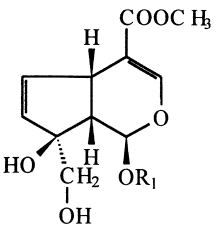
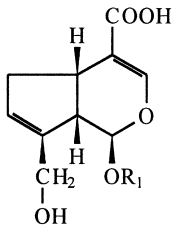
0021-9673/98/$19.00
PII S0021-9673(97)01227-2



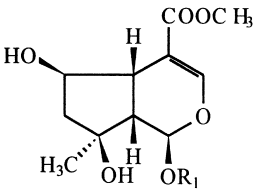
geniposide (GP)



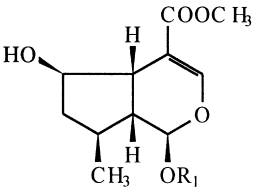
gardenoside (GD)



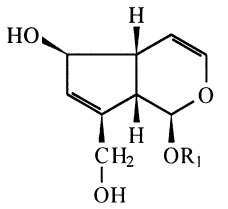
geniposidic acid (GPA)



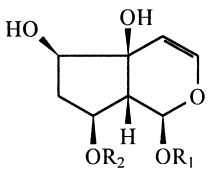
shanzhiside (SH)



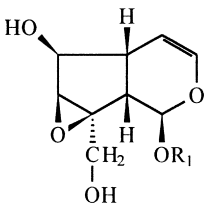
loganin (LO)



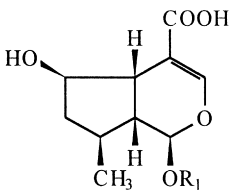
aucubin (AU)



harpagoside (HR)



catalpol (CT)



loganic acid (LOA)

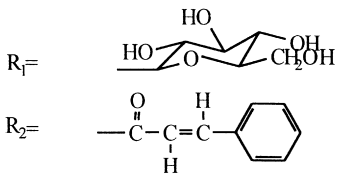


Fig. 1. Structures of the nine iridoids.

Yoneyama (Osaka, Japan), loganin (LO), shanzhiside methylester (SH), harpagoside (HR) and loganic acid (LOA) from Extrasynthese (Genay, France) and gardenoside (GD) from Wako (Osaka, Japan). α-Cyclodextrin (α-CD), β-cyclodextrin (β-CD) and hydroxyethyl-β-cyclodextrin (HE-β-CD) were obtained from Aldrich (Milwaukee, WI, USA), γ-cyclodextrin (γ-CD), 2,6-di-O-methyl-β-cyclodextrin (DM-β-CD), 2,3,6-tri-O-methyl-β-cyclodextrin (TM-β-CD) and sodium borate from Nacalai Tesque (Kyoto, Japan), methyl-β-cyclodextrin (M-β-CD) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) from Sigma (St. Louis, MO, USA), potassium dihydrogenphosphate from Kanto (Tokyo, Japan). Methanol and acetonitrile were of LC grade (Mallinckrodt, KY, USA) and phosphoric acid was of analytical-reagent grade (Merck, Darmstadt, Germany). Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all buffer and sample solutions.

2.2. Apparatus and conditions

2.2.1. CE system

The analysis was carried out on a Hewlett-Packard (HP^{3D} CE) capillary electrophoresis system equipped with a photodiode array detector (210 nm and 230 nm) and a 90 cm×75 μm I.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA) with the detection window placed 81.5 cm from injection. The running conditions were as follows: injection mode, 50 mbar for 3 s injected sample solution and then 50 mbar for 3 s injected deionized water; applied voltage 16 kV (constant voltage, positive to negative polarity); and cartridge temperature 30°C. The electrolyte was a buffer solution that contained 50 m*M* $Na_2B_4O_7$ and 30 mg/ml DM-β-CD; run time was 32 min.

At the beginning of experimentation each day, the capillary was purged with 0.5 *M* NaOH for 5 min, followed by 0.1 *M* NaOH for 5 min, deionized water for 5 min and then running buffer for 5 min. Between runs, the capillary was flushed with 0.1 *M* NaOH for 2 min followed by deionized water 2 min and then running buffer 10 min.

2.2.2. HPLC system

HPLC was performed on a Shimadzu LC-10AD apparatus equipped with a Shimadzu CBM-10A system controller and a Shimadzu SPD-M10A photodiode array detector (210 nm and 230 nm). Satisfactory separation of the nine iridoids was obtained with a reversed-phase column (Cosmosil 5 C_{18}-AR, 5 μm, 25 cm×4.6 mm I.D.; Nacalai Tesque) eluted at a flow-rate of 0.8 ml/min with a linear solvent gradient of A–B [A=an aqueous solution consisting of 20 m*M* KH_2PO_4; B=CH_3CN] varying as follows: 0 min, 96:4; 30 min, 80:20; 40 min, 25:75.

3. Results and discussion

3.1. Analytical conditions of CE method

In the beginning, the detection wavelength was set at 230 nm for the nine iridoids, but this wavelength was not the maximum absorption band of HR, CT and AU which do not have the conjugated carbonyl groups on their rings. To avoid the spikes caused by detecting at 200 nm, we set the wavelength at 210 nm and 230 nm at the same time to detect all nine iridoids.

Buffer solutions of borate and phosphate with different pH values were applied to separate the nine compounds in our preliminary trials. Alkali solutions were preferable for separation, therefore an adequate borate concentration was considered first. However, GP is neutral and was eluted with electroosmotic flow (EOF) in a series of different borate concentrations, and LO and SH and AU and CT overlapped completely under these conditions. Different kinds of surfactants such as sodium dodecyl sulfate (SDS), sodium cholate (SC) and lauryltrimethylammonium chloride (LTAC) were added to buffers, but similar results were obtained. On second thoughts, cyclodextrins with different ring-sizes or different substituted groups were chosen for their high selectivity.

Addition of various cyclodextrins to a borate buffer, gave the resolutions and the number of theoretical plates for some iridoids shown in Table 1 and the electropherograms are shown in Fig. 2. The data in Table 1 show that no matter which cyclodextrin, α-, β- or γ-CD, was added, it did not affect the poor resolution of the GP–LO, LO–SH and SH–AU pairs. Therefore, several β-CD derivatives,

Table 1
Resolutions and theoretical plate numbers of iridoids with various cyclodextrins

Cyclodextrins[a]	Resolution					Number of theoretical plates($\cdot 10^5$)			
	GP–LO	LO–SH	SH–AU+CT[b]	HR–GD	GPA–LOA	HR	GD	GPA	LOA
No CD	0	0	0	1.42	1.12				
α-CD	0	0	0	3.24	1.25				
γ-CD	0	0	0	5.21	1.38				
β-CD	0	0	0	1.45	7.31	1.38	3.14	3.99	1.98
HE-β-CD	1.41	0	0.73	6.39	5.56	3.21	3.51	3.27	2.28
HP-β-CD	1.27	0	0	7.19	7.62	2.94	3.26	1.89	2.07
M-β-CD	2.32	1.18	1.24	5.21	2.65	1.72	2.56	2.36	2.46
DM-β-CD	1.96	1.91	1.63	2.22	2.21	1.40	3.25	2.29	2.35
TM-β-CD	0.81	0	0	8.99	1.27	2.45	2.81	3.67	2.02

[a] Buffer solution containing 50 m*M* $Na_2B_4O_7$ and 30 mg/ml of cyclodextrins.
[b] Resolution between shanzhiside and aucubin and catapol; AU and CT overlapped completely.

HE-β-CD, HP-β-CD, M-β-CD, DM-β-CD and TM-β-CD, were tried, and the results indicated that cyclodextrins with higher polar substituents (HE-β-CD, HP-β-CD) separated carboxylic acids well ($R_s >$ 55.6), but good separation of other compounds was not obtained. M-β-CD, DM-β-CD and TM-β-CD with lower polar substituents showed better resolutions for GP–LO and SH–AU pairs. In order to separate all the compounds, DM-β-CD was chosen. The separation of the neutral compounds in the buffer that contained borate and DM-β-CD might be due to the degree of association among borate, dextrin and the compounds.

DM-β-CD could separate all the compounds except AU and CT. The only difference in structure between AU and CT is at the C7–C8 position, the former has a double bond and the latter possesses an epoxide ring. In order to separate the two compounds a number of organic solvents (methanol, ethanol, propanol, acetonitrile, butyronitrile, valeronitrile and hexanenitrile) and/or additives (urea, valine and Ba^{2+}) were added. However, none of them were found to be effective. Efforts to change their structure by reaction with bases or acids were tried previously, but were found to be unsuccessful.

3.1.1. Effect of borate concentration

Electrolyte systems at different $Na_2B_4O_7$ concentrations (20–80 m*M*) were used in order to study the effect of borate concentration on the separability. In Fig. 3, the migration times for the individual iridoids obtained at different borate concentrations are shown. The migration times of all compounds increased with the increase of borate concentrations. Among them, those of GPA and LOA which have a carboxylic group and would be deprotonated much more easily as the pH increases, increased most rapidly. GP eluted with the EOF under these CZE conditions. The increases in migration time for LO, SH, AU and CT closely paralleled that of the EOF. LO, SH, AU and CT completely overlapped when the series of borate solutions was used. With a 1,2-diol group, HR and GD, which could form a tighter five-membered-ring transition state with boron at higher borate concentrations, moved with migration times intermediate between those of the acids (GPA and LOA) and the others. When the borate concentration was lower than 50 m*M*, GPA and LOA and the AU–CT and LO–SH pairs partially overlapped. With concentration higher than 50 m*M*, the peaks of HR and GD gradually moved together. A buffer solution with 50 m*M* borate was found to be the best.

3.1.2. Effect of DM-β-CD concentration

Electrolyte systems containing 50 m*M* $Na_2B_4O_7$ at different 2,6-di-O-methyl-β-cyclodextrin concentrations ranging from 0 to 30 mg/ml were tried to study the effect of DM-β-CD on the separability. The results obtained are given in Fig. 4. Generally speaking, the migration times of all compounds became longer as DM-β-CD concentration increased, and the sequences of GD–HR and GPA–LOA changed after adding DM-β-CD. A concentration of

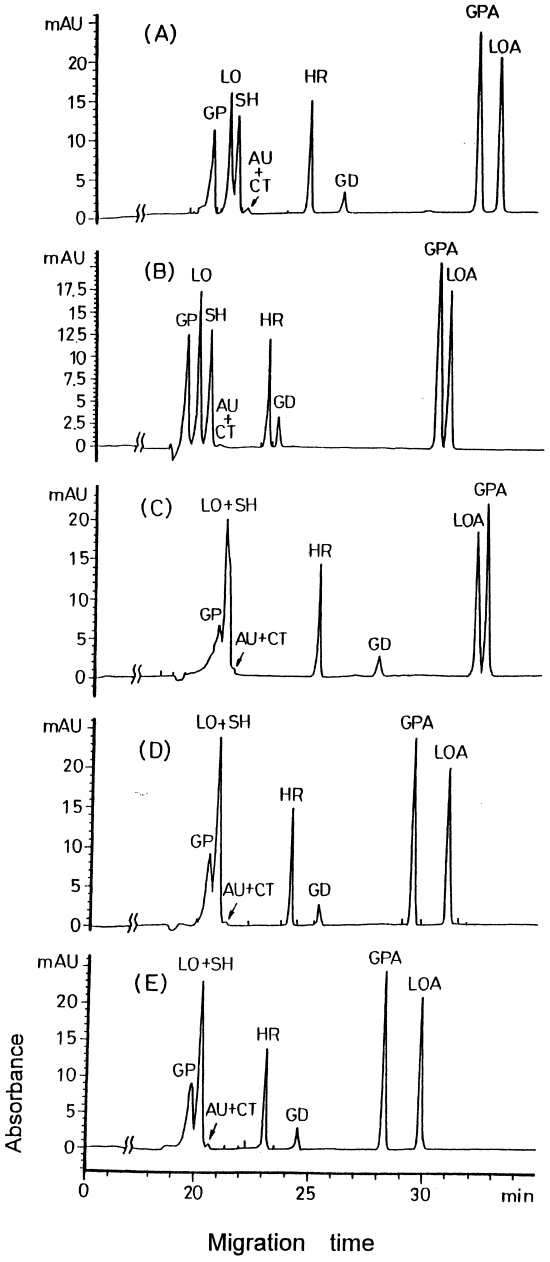


Fig. 2. Capillary electropherograms of a mixture of the nine iridoids with different β-CD derivatives (30 mg/ml): (A) M-β-CD, (B) DM-β-CD, (C) TM-β- CD, (D) HE-β-CD and (E) HP-β-CD.

15 mg/ml could separate all the compounds, but higher DM-β-CD concentration could improve the separability of GPA and LOA. The resolution values

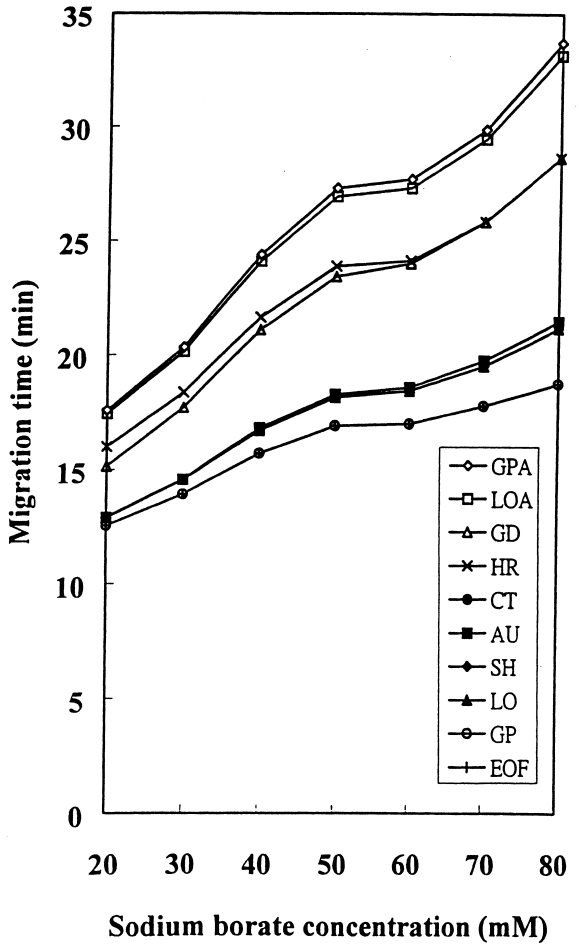


Fig. 3. Effect of $Na_2B_4O_7$ concentration on migration time. All these experiments were conducted at a voltage of 16 kV across the 90 cm×75 μm I.D. separating tube filled with buffers of different borate concentrations; temperature 30°C. Symbols as in Fig. 1.

between them were 0.24, 1.39, 1.24 and 2.21 at 15, 20, 25 and 30 mg/ml, respectively. As a result, 30 mg/ml was chosen. However, the separation of AU and CT could not be achieved, because of their similarity in structure. The final electropherograms of the nine iridoids running with an electrolyte containing 50 m*M* $Na_2B_4O_7$ and 30 mg/ml DM-β-CD are shown in Fig. 5. The migration times of the iridoids were: geniposide, 19.5 min; loganin, 20.0 min; shanzhiside, 20.5 min; aucubin, 21.0 min; catapol, 21.0 min; harpagoside, 23.3 min; gardenoside, 24.0 min; geniposidic acid, 30.4 min and loganic acid, 30.9 min.

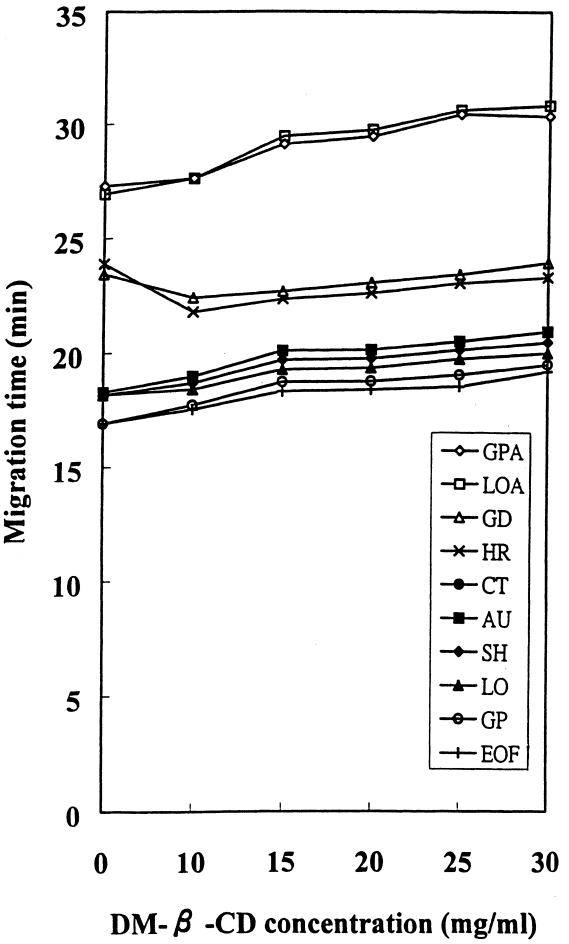


Fig. 4. Effect of DM-β-CD concentration on migration time. The carriers were 50 m*M* $Na_2B_4O_7$ at different DM-β-CD concentrations. Other conditions as in Fig. 3.

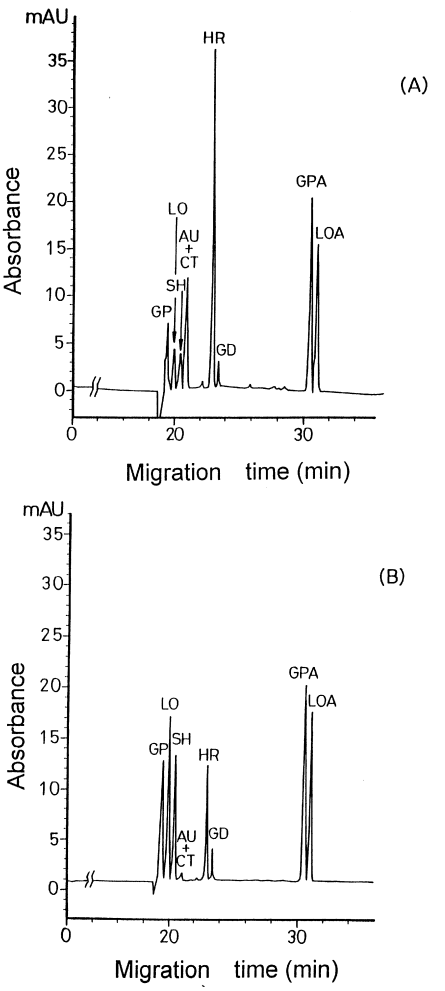


Fig. 5. Capillary electropherograms of a mixture of the nine iridoids (A) detection at 210 nm and (B) detection at 230 nm.

3.2. Analytical conditions of HPLC method

In our laboratory, the glycosides in ginseng [9] and *Paeoniae radix* [10] had been well separated with reversed-phase HPLC using phosphate solution as the eluent; therefore, addition of potassium dihydrogenphosphate to the mobile phase and adjustment of pH values with phosphoric acid were the first determinant factor concerned. Methanol and acetonitrile in different ratios were also studied for enhancement of separation.

3.2.1. Effect of dihydrogenphosphate concentration

KH_2PO_4 buffer solutions at different concentrations ranging from 0 to 50 m*M* were tried. In Fig. 6, the capacity factors (k') of the nine compounds obtained at different KH_2PO_4 concentrations are shown. It is known that the presence of phosphate cannot only change the ionic strength of the mobile phase but also vary the situation of end capping of a column [11]. Hence, as the phosphate concentration increased, the retention times shifted and the peak widths were also narrowed for most of the compounds.

When the KH_2PO_4 concentrations were higher than 20 m*M*, separation of the nine compounds showed no more improvement; when the concen-

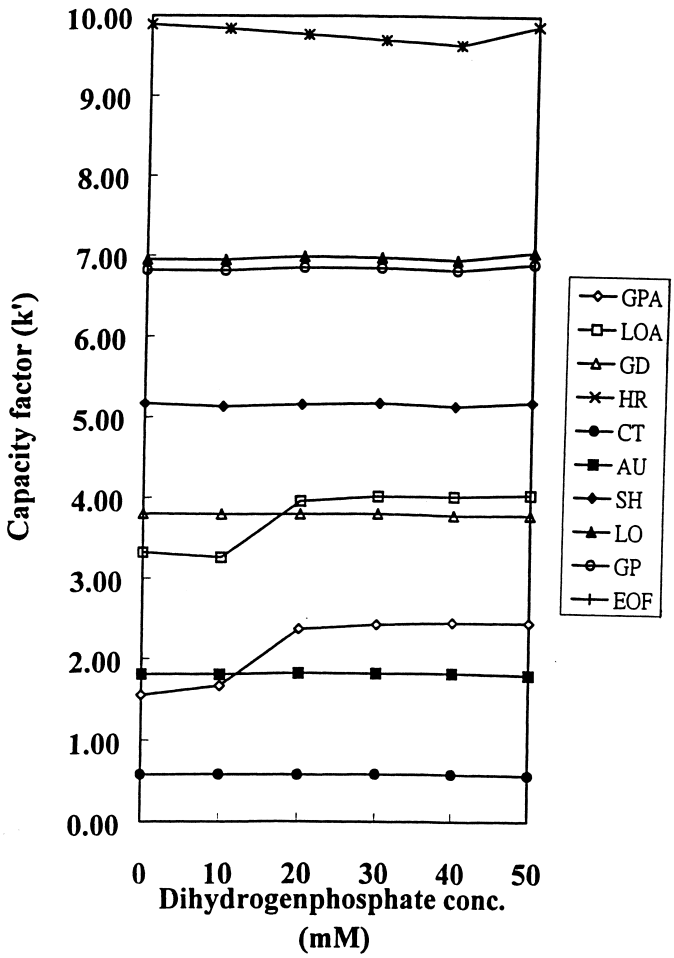


Fig. 6. Effect of KH_2PO_4 concentration on capacity factor. All results were obtained with a reversed-phase column (Cosmosil $5C_{18}$-AR, 5 μm, 25 cm×4.6 mm I.D.) eluted at a flow-rate of 0.8 ml/min with a linear solvent gradient of A–B [A=aqueous solution of 20 m*M* KH_2PO_4; B=CH_3CN] varying as follows: 0 min, 96: 4; 30 min, 80:20; 40 min, 25:75.

trations were lower than 20 m*M*, AU and GPA could not be separated totally. To avoid the possible column damage caused by high salt concentration and to obtain better resolutions, a buffer solution containing 20 m*M* KH_2PO_4 was subsequently tried and chosen.

3.2.2. Effect of pH value

In order to investigate the relationship between pH value and separability, four buffer systems containing 20 m*M* KH_2PO_4 were used, which contained added H_3PO_4 to obtain pH values ranging from 3.11 to 4.43. Higher pH values caused easier deprotonation and resulted in higher polarity of LOA and GPA. So the higher the pH values were, the shorter the retention times of these two acids became. For the other compounds, there was not much difference in either retention time or resolution. Therefore, a buffer of 20 m*M* KH_2PO_4 with no H_3PO_4 (pH 4.43) was chosen.

Methanol and acetonitrile have an almost equal scale in polarity but possess a quite different ability in column scouring. Varying the ratios of methanol and acetonitrile in the organic portion of the mobile phase has also been tried. As methanol concentration increased, the retention times of all compounds

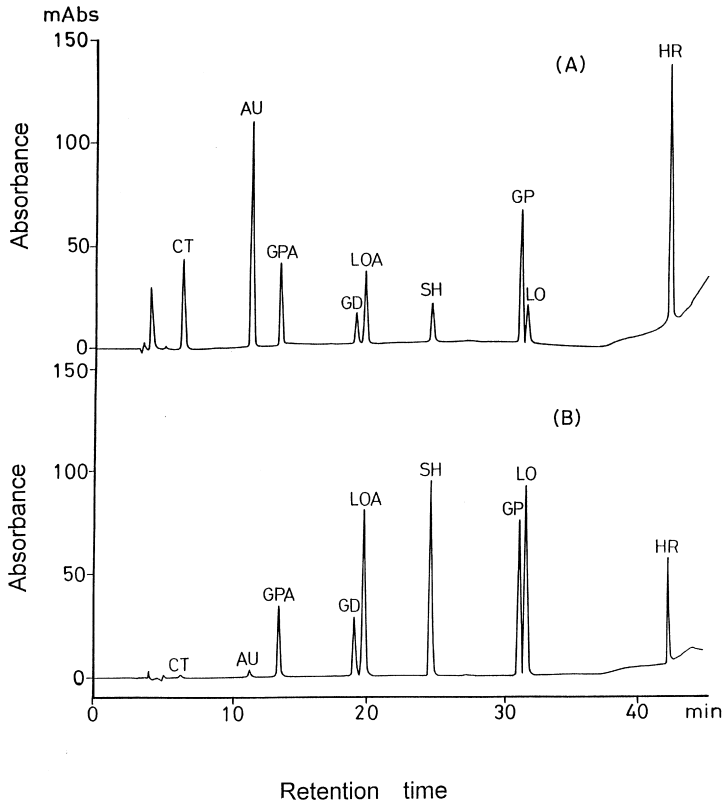


Fig. 7. HPLC chromatograms of a mixture of the nine iridoids (A) detection at 210 nm and (B) detection at 230 nm.

became longer, especially the two acids, GPA and LOA. The sharpness of peaks was not significantly changed by adding methanol. However, the higher the methanol concentration was, the more obvious a baseline drift became, especially at a low wavelength (200 nm). To shorten the analysis time and avoid the baseline fluctuation, 100% acetonitrile was used in pump B.

From the above results, the best resolution was obtained with an eluent containing 20 m*M* potassium dihydrogenphosphate in pump A and acetonitrile in pump B. Fig. 7 shows the separation of the nine iridoids with the retention times: catapol, 6.17 min; aucubin, 11.1 min; geniposidic acid, 13.2 min; gardenoside, 18.8 min; loganic acid, 19.4 min; shanzhiside, 24.2 min; geniposide, 30.9 min; loganin, 31.5 min; and harpagoside, 42.3 min.

3.3. Comparison of the CE and HPLC methods

Generally speaking, HPLC can totally separate the nine iridoids, whereas CE cannot separate AU and CT. However, CE has better analytical efficiency for most compounds (Table 2) such as LO, SH, GD, GP and LOA, and is quicker. The analysis time for CE

Table 2
Number of theoretical plates ($\cdot 10^4$) for the various compounds analyzed with CE and HPLC

Method	GP	LO	SH	HR	GD	GPA	LOA	CT	AU
CE	6.89	9.89	10.4	29.5	30.5	22.7	23.4	[a]	[a]
HPLC	10.8	7.11	5.32	51.2	2.61	1.58	4.38	0.382	1.39

[a] Inseparable.

was 32 min which is shorter than the 45 min required of HPLC.

Inasmuch as the baseline has great bearing on assay results, it becomes an important consideration to avoid baseline drift for an analytical method. The commonly used additives such as methanol, acetic acid, etc. and the gradient elution employed in the HPLC analysis all affect the baseline stability to a considerable degree. In this experiment, because methanol has absorption at wavelengths below 220 nm which would cause interference to detection, we could only use the far-UV grade acetonitrile, and even by so doing, the baseline drifts still occurred.

The greatest influence on the analysis mechanism in CE is the ratio of electric charge to mass of the analysate. In an alkaline solution, LOA and GPA were present in a completely dissociated state, and hence had a longer migration time; HR and GD formed complexes with boron owing to the 1,2-diol in their structures, and thus had a next longest migration time; and the neutral compounds, GP, LO, SH, AU and CT, almost eluted with the EOF but could be separated when DM-β-CD was added. However, AU and CT could not be separated, probably because they were equally firmly entrapped in DM-β-CD. In the reversed-phase HPLC, the polarity of the compound analyzed has very close relationship with its retention time. Therefore, CT and AU can be separated very easily with HPLC. As for LOA and GPA, the pH values affect their dissolution rates and hence also their retention times.

Application of these techniques to Chinese herb drugs and Chinese herb preparations is being studied.

Acknowledgements

Financial support from the National Science Council, Taiwan, is gratefully acknowledged (NSC 86-2113-M-003-010).

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