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Capillary electrophoretic determination of the constituents of Paeoniae Radix

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Abstract

A method combining the techniques of capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) has been developed to separate a total of eight peony constituents. The CZE method was used to determine the content of paeonol, oxypaeoniflorin, benzoic acid, pentagalloylglucose and gallic acid, and MEKC technique based on sodium cholate was applied to analyze albiflorin, paeoniflorin, benzoylalbiflorin, paeonol and oxypaeoniflorin. Linearity around two orders of magnitude of concentration was generally obtained and limits of detection for these compounds were in the range of 2.6–23.7 μg/ml. The relative standard deviations of migration times were less than 1.43% ($n=6$). Contents of peony constituents in an ethanol–water extract of *Paeonia lactiflora* Pall. sample could easily be determined by this method.

Keywords: Paeoniae Radix; Peony constituents

1. Introduction

Paeoniae Radix is the dried root of *Paeonia veitchii* Lynch or *P. lactiflora* Pall. (=*P. albiflora* Pall.) and is a commonly used Chinese herb drug possessing the effects of cleansing heat, cooling blood and invigorating blood circulation, etc. [1]. The drug is derived from a ranunculaceous plant known to contain oxypaeoniflorin (OPF) [2–4], albiflorin (AF), paeoniflorin (PF) [2–6], gallic acid (GA), pentagalloylglucose (PG) [7], benzoylalbiflorin (BAF), paeonol (PN) and benzoic acid (BA) (Fig. 1) as its bioactive constituents [8–10]. Several methods have been used to determine one to four of these constituents contained in the crude drug, such as thin-layer chromatography (TLC) [11], high-performance liquid chromatography (HPLC) [12] and capillary zone electrophoresis (CZE) [13].

Capillary electrophoresis is a widely applied technique in separation science on account of its high efficiency, rapid rate of separation and small sample requirment and offers satisfactory results in the analysis of some Chinese herbs [14–17]. Here we used CZE to determine the contents of PN, OPF, BA, PG and GA and used MEKC to measure the amount of AF, PF, BAF, OPF and PN. The effects of borate concentration and pH values of the electrolyte in CZE and the effects of surfactant and methanol

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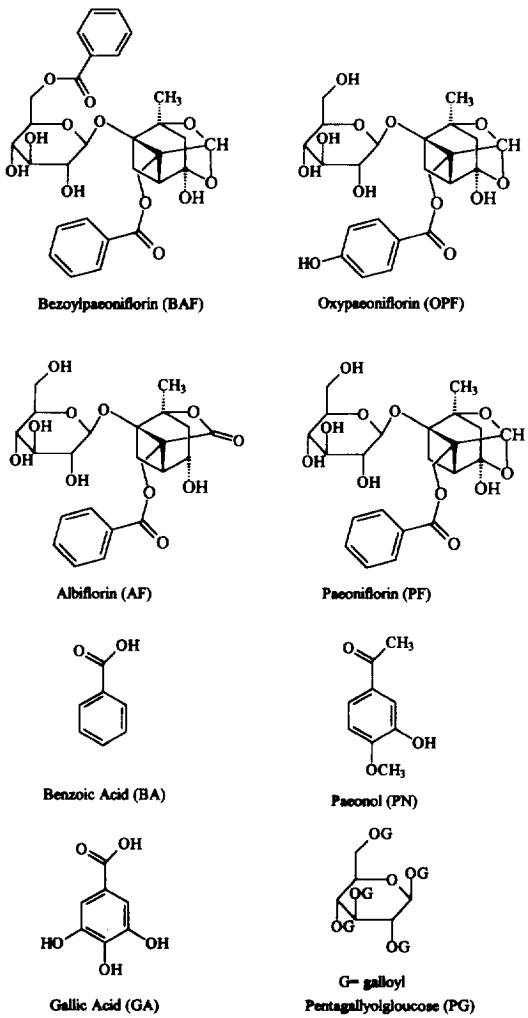

Fig. 1. Structures of the eight constituents in Paeoniae Radix.

concentration of the carrier in MEKC on the migration behaviour of the solutes were studied.

2. Experimental

2.1. Reagents and materials

Oxypaeoniflorin [2–4], albiflorin, paeoniflorin [2–6], pentagalloylglucose [7] and benzoylalbiflorin were isolated from Paeoniae Radix; paeonol was isolated from *Paeonia moutan* Sims [1]. The structures of these isolated standards were elucidated on the basis of spectra data, such as IR, PMR, CMR and MS and their purity was checked by HPLC. Gallic acid and benzoic acid were purchased from Merck (Darmstadt, Germany). Sodium cholate (SC) was purchased from Sigma, (St. Louis, MO, USA), sodium borate, ammonia solution (28%) and γ-cyclodextrin from Nacalai Tesque (Kyoto, Japan), sodium dihydrogenphosphate from Kanto (Tokyo, Japan), β-cyclodextrin and naringin hydrate from Aldrich (Milwaukee, WI, USA). Methanol and acetonitrile of LC grade were obtained from Mallinckrodt (Paris, KY, USA). Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all buffer and sample solutions. Paeoniae Radix was purchased from the Chinese herbal market in Taipei (Taiwan).

2.2. Preparation of Paeoniae Radix extract

A 1.0-g sample of pulverized Paeoniae Radix was extracted by refluxing with 50% ethanol (7 ml) for 15 min and then centrifuging at 1500 *g* (Universal, Hettich Zentrifugen) for 5 min. The extraction was repeated three times. The extracts were combined and filtered through a No. 1 filter-paper. After the addition of 5 ml internal standard solution (1.25 mg of naringin hydrate in 1 ml of 50% ethanol), the Paeoniae Radix extract was diluted to 50 ml with 50% ethanol. This solution was passed through a 0.2-μm filter before injection into the capillary electrophoresis system.

2.3. Apparatus and conditions

The analysis was carried out on a Hewlett–Packard (HP 3D) capillary electrophoresis system equipped with a photodiode array detector operating at 230 nm and with 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, 25 kV (constant voltage, positive to negative polarity); and cartridge temperature 30°C. The separation was achieved by using CZE and MEKC techniques. In CZE, the electrolyte was a buffer solution that contained 15

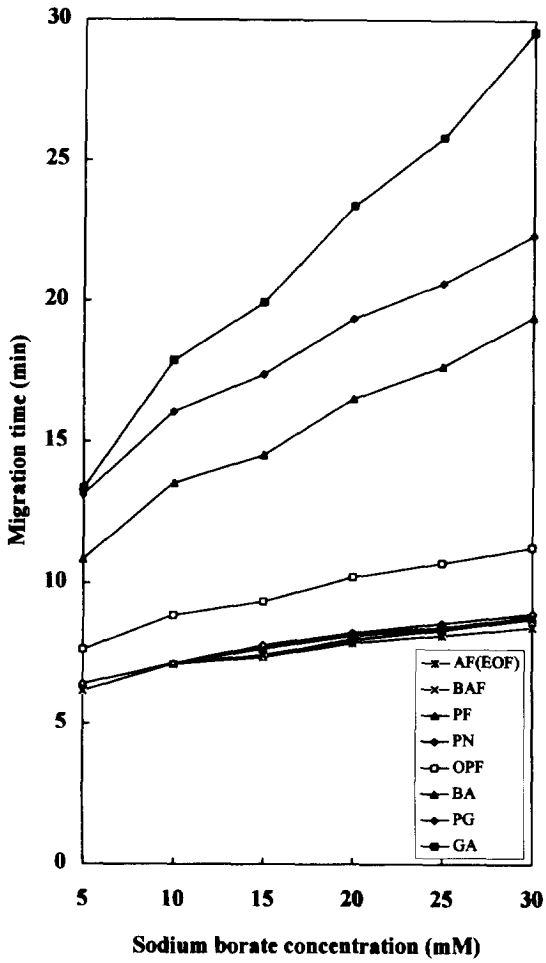

Fig. 2. Effect of $Na_2B_4O_7$ concentration on migration time. All these experiments were conducted at a voltage of 25 kV across the 90 cm×75 μm I.D. separating tube filled with buffers of different borate concentrations; temperature 30°C; detection wavelength 230 nm. Symbols are the same as those in Fig. 1.

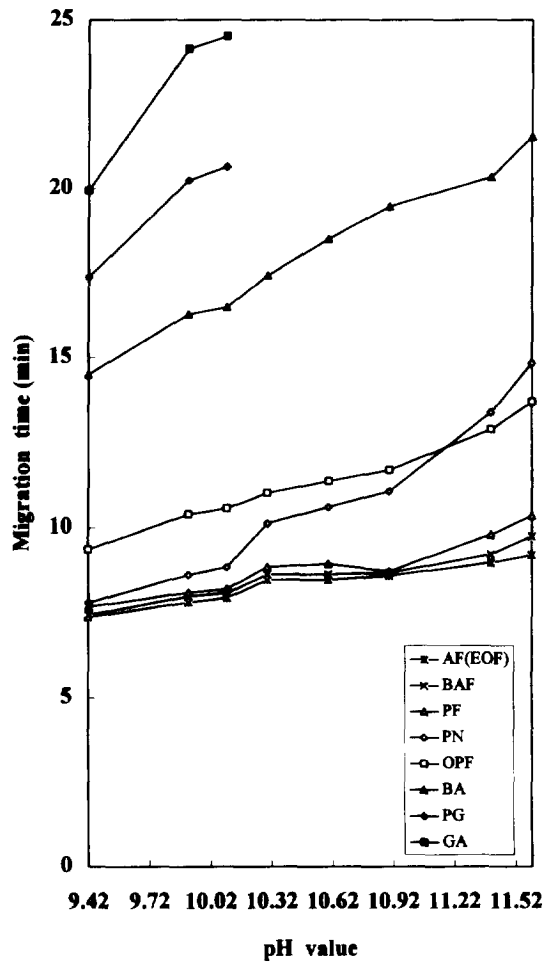

Fig. 3. Effect of pH on migration time. The carriers were 15 m*M* $Na_2B_4O_7$ at different pH values (adjusted with ammonia solution). Other conditions are the same as those in Fig. 2.

m*M* $Na_2B_4O_7$ and adjusted to pH 9.8 with ammonia solution; run time 25 min. In MEKC, the electrolyte was a buffer solution consisting of 15 m*M* $Na_2B_4O_7$ (pH 9.8, adjusted with ammonia solution) and 50 m*M* SC–methanol (9:1).

At the beginning of experimentation each day, 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 with running buffer for 5 min. Between the runs, the capillary was flushed with 0.5 *M* NaOH for 2 min followed by deionized water 6 min and then with running buffer 5 min. The CZE and MEKC were performed by using a single capillary tube.

3. Results and discussion

3.1. Analytical conditions

A buffer solution of 100 m*M* $Na_2B_4O_7$ (pH 10.5) was applied to separate the eight constituents mentioned above in our preliminary trials. However, this Honda's method [13] not only limited the separation to just three compounds (PF, OPF and GA) but also

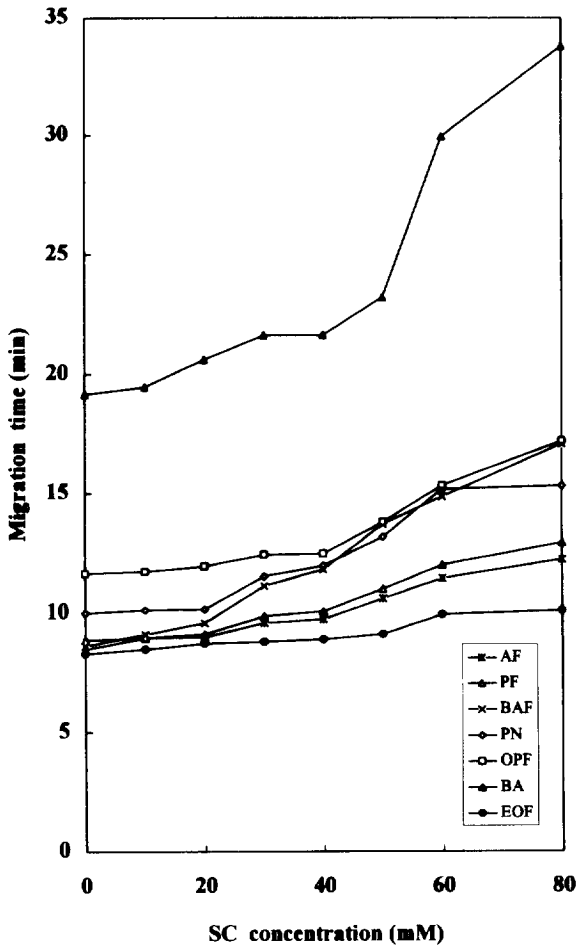

Fig. 4. Effect of SC concentration on migration time. The carriers were 15 m*M* $Na_2B_4O_7$ (adjusted to pH 9.8 with ammonia solution) containing 0–80 m*M* SC. Other conditions are the same as those in Fig. 3.

caused a high electric current and a baseline fluctuation owing to the high buffer concentration. Thus, an adequate borate concentration was first to be considered. After a series of trials, a buffer containing 15 m*M* $Na_2B_4O_7$ (adjusted to pH 9.8 with ammonia solution) was found to be able to separate the monoterpene glycosides (PF, AF, OPF and BAF) from the others (PN, BA, PG and GA) successfully. Under this CZE condition, glycosides with similar structures (PF, AF and BAF) still overlapped completely. Efforts to try to improve their separation by changing the pH values were found to be invalid. Therefore, MEKC mode was used to separate these three glycosides by adding different compounds such as SDS, β-cyclodextrin, γ-cyclodextrin and SC to the borate solution. Only β-cyclodextrin and especially SC gave a significant improvement of resolution. Methanol and acetonitrile were also used as organic modifiers to make the peaks sharper and to produce a better separation and methanol was found to give better results in the separation of OPF from an unidentified component of the crude drug extract.

Differences in sample zone and running buffer conductivities can effectively skew the peak shapes

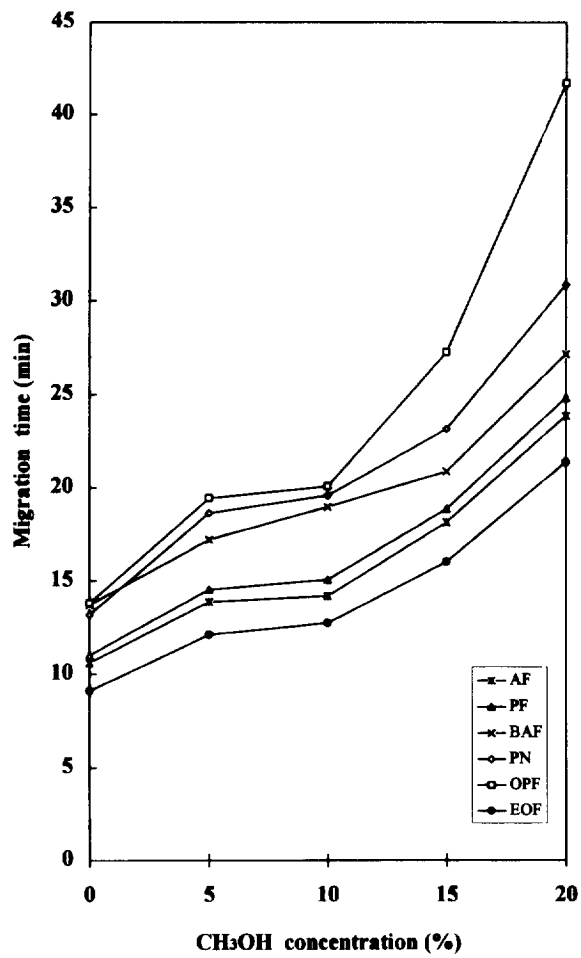

Fig. 5. Effect of methanol concentration on migration time. The carriers were buffer solution (15 m*M* $Na_2B_4O_7$ with pH 9.8 and 50 m*M* SC) mixing with different amounts of methanol. Other conditions are the same as the those in Fig. 4.

[18]. When the solute zone has a lower mobility than the running buffer, the leading edge will be sharp and the trailing edge diffuse. To avoid such a peak distortion and equalize the conductivities, the optimum injection mode was set at 3-s injected sample solution and then 3-s injected deionized water.

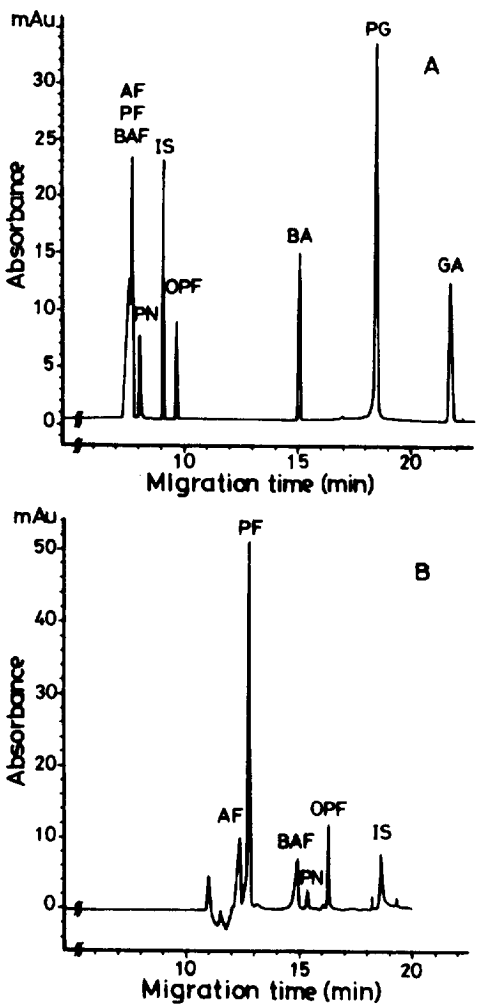

Fig. 6. Capillary electropherograms of a mixture of the eight authentic standards in (A) CZE, the electrolyte was a buffer solution consisting 15 m*M* $Na_2B_4O_7$ and adjusted to pH 9.8 with ammonia solution, and (B) MEKC, 15 m*M* $Na_2B_4O_7$ (pH 9.8, adjusted with ammonia solution) and 50 m*M* SC–methanol (9:1). Peaks: AF, 0.150 mg/ml; PF, 0.445 mg/ml; BAF, 0.072 mg/ml; PN, 0.008 mg/ml; OPF, 0.167 mg/ml; BA, 0.038 mg/ml; PG, 0.139 mg/ml; GA, 0.033 mg/ml; I.S.=naringin hydrate, 0.126 mg/ml.

3.1.1. CZE

All constituents except for PF, AF and BAF were successfully determined in a single run by CZE under suitable conditions. The separation was achieved by optimizing the sodium borate concentration and the pH of the carrier.

Effect of sodium borate concentration

Electrolyte systems at six different $Na_2B_4O_7$ concentrations (5–30 m*M*) were used in order to study the effect of borate concentration on the separability. In Fig. 2, the migration times for the constituents obtained at different borate concentrations are shown. The migration times of these eight compounds, especially those of BA, PG and GA, increased with the increase of borate concentration of the buffer. Totally, BA, PG and GA moved much slower than the others owing to the easiness of deprotonating of the carboxylic or the phenolic group and the affinity of glucosyl moiety with the borane atoms. When the concentrations were lower than 12 m*M*, AF, BAF, PF and PN moved with electroosmotic flow (EOF). From the results, a buffer solution at borate concentration higher than 15 m*M* was found to be necessary to produce a good resolution. To avoid thermal diffusion and obtain shorter run time, 15 m*M* borate was found to be the best.

Effect of pH value

Electrolyte systems containing 15 m*M* $Na_2B_4O_7$ at eight different pH values ranging from 9.42 to 11.52 (adjusted with ammonia solution) were used to study the effect of pH on the separability. The results obtained are shown in Fig. 3, where the migration times are plotted against pH values. There was an increase in migration times of the constituents, especially those of GA, PG and BA, when the pH value in the electrophoretic solution increased. At the pH values higher than 10, PG and GA could not be detected within 50 min and the peak-heights of AF, PF, BAF and OPF were found to reduce markedly or even disappear. The latter phenomenon might be due to the hydrolysis of ester linkage in these conditions. Fig. 3 showed that a buffer solution at pH 9.42 or 9.80 was able to produce a good resolution. However the resolution values between BAF and PN were 1.52 at pH 9.42 and 6.18 at pH 9.80 and the peaks of

Table 1
Data for linear ranges, correlation coefficients and detection limits

Constituents	CZE					MEKC				
	Linear range (mg/ml)	Slope ($\times 10^2$)	Intercept	r	Detection limit (μg/ml, $S/N=3$)	Linear range (mg/ml)	Slope ($\times 10^2$)	Intercept	r	Detection limit (μg/ml, $S/N=3$)
AF						0.028–0.450	0.038	0.115	0.9991	10.1
PF						0.036–1.630	0.057	0.151	0.9992	18.3
BAF						0.012–0.216	0.116	0.042	0.9990	23.7
PN	0.008–0.025	0.123	0.010	0.9995	3.8	0.008–0.025	0.178	0.017	0.9993	5.6
OPF	0.011–0.500	0.027	0.006	0.9997	11.2	0.028–0.500	0.031	0.038	0.9996	22.4
BA	0.006–0.113	0.314	0.006	0.9998	2.6					
PG	0.009–0.418	0.406	0.049	0.9987	4.5					
GA	0.005–0.099	0.561	0.038	0.9998	2.8					

BAF, PF and AF could partially be separated at pH 9.80 as well. Therefore, a solution of 15 m*M* $Na_2B_4O_7$ with pH 9.80 (adjusting with ammonia solution) was chosen. Under this condition, PN, OPF, BA, PG and GA were successfully separated, but PF, BAF and AF were partially overlapped.

3.1.2. MEKC

The subsequent MEKC method was performed in order to obtain a well-separated electropherogram for AF, PF and BAF. In this technique, SC was used as surfactant to produce a desirable separation and methanol was added as organic modifier to improve the peak resolution and theoretical plate numbers. Under these conditions, AF, PF, BAF, PN and OPF could be separated successfully.

Effect of SC concentration

Electrolyte systems containing 15 m*M* $Na_2B_4O_7$ and ammonia solution (adjusted to pH 9.8) at eight different SC concentrations ranging from 0 to 80 m*M* were used to study the effect of SC concentration on separation. The results obtained are given in Fig. 4. As the SC concentration increased, both the migration times of all constituents and the resolution values between AF and PF increased, but the resolution values between OPF and PN decreased. When SC was absent or at a concentration lower than 20 m*M*, AF, PF and BAF overlapped completely or partially with EOF. At 30, 40 and 50 m*M* SC, the resolution values were 1.40, 1.54 and 2.48 between AF and PF and were 1.26, 0.728 and 0.612 between OPF and PN. Although these two sets of values were totally contrary, it was found that the addition of methanol to the buffer could greatly improve the resolution between OPF and PN but had almost no influence on that between AF and PF. As a result, the borate buffer solution consisting of 50 m*M* SC was chosen.

Effect of methanol concentration

Electrolyte systems containing 15 m*M* $Na_2B_4O_7$ (adjusted with ammonia solution to pH 9.8) and 50 m*M* SC at five different methanol concentrations ranging from 0 to 20% were used to study the effects of methanol concentration on separation. The results obtained are given in Fig. 5. As methanol concentration increased, the migration times of all compounds became longer and the resolutions of all peaks became better. However, the influence on AF and PF separation was less dramatic than that of BAF, PN and OPF. A buffer solution with the presence of 5 or 10% methanol was found to produce a good resolution. Although the former concentration provided larger differences in migration time between BAF and PN (1.52 min at 5% and 1.14 min at 10% methanol), but the latter gave higher theoretical plate numbers for BAF (4.0×10^4 at 5% and 4.7×10^4 at 10% methanol). From the above results, the best resolution was obtained with an electrolyte with 90% of buffer solution (15 m*M* $Na_2B_4O_7$ with pH 9.8 and 50 m*M* SC) and 10% of methanol. Under these conditions, AF, PF, BAF, PN and OPF could be determined successfully. By the way, BA, PG and

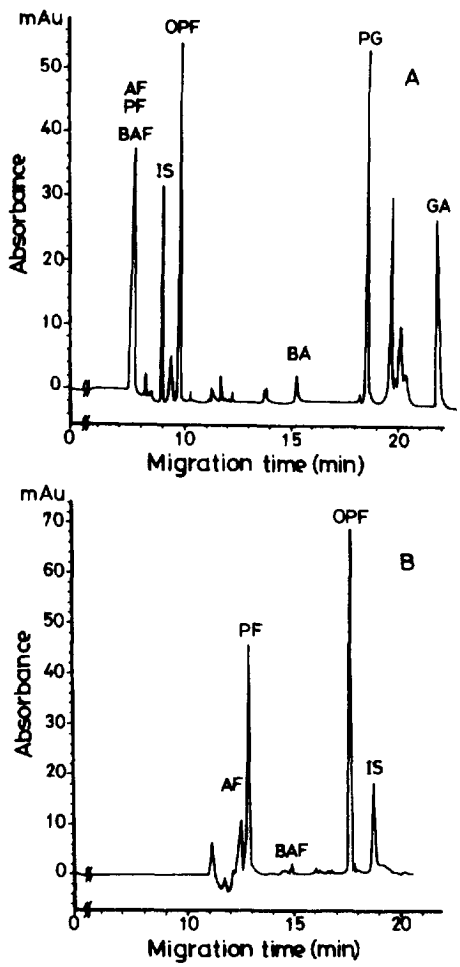

Fig. 7. Capillary electropherograms of the extract of a Paeoniae Radix (*Paeonia lactiflora*) sample in (A) CZE and (B) MEKC. Peaks and the electrolytes as in Fig. 6.

GA could not be detected within 50 min when this MEKC method was applied.

The electropherograms showing the separation of a mixture of the eight authentic standards are shown in Fig. 6A and B when CZE and MEKC were applied respectively.

3.2. Calibration graphs for the constituents

Calibration graphs for the constituents were obtained over the ranges 0.005–0.500 mg/ml in CZE and 0.008–1.630 mg/ml in MEKC. The linear ranges, regression equations of the standard curves, correlation coefficients and detection limits for the eight compounds are shown in Table 1. The results showed good linear relationship between peak-area ratios and concentration. The detection limits (S/N= 3) for these constituents were 2.56–23.7 μg/ml (0.04–0.37 ng, column I.D. 75 μm).

3.3. Determination of the constituents in Paeoniae Radix

When the test solution was analysed by CE under these selected conditions, the electropherograms shown in Fig. 7A and B were obtained and the contents of the constituents in Paeoniae Radix (three *Paeonia lactiflora* samples) were calculated: OPF, 0.06±0.03 (in CZE), 0.07±0.04 (in MEKC); BA, 0.02±0.01; PG, 2.04±0.10; GA, 0.29±0.01; AF, 5.10±0.13; PF, 18.70±0.51; BAF, 0.36±0.01 mg/g (mean±S.D.; n=3); PN was below the detection limit.

Table 2
Recovery and reproducibility of migration time (t_m) and amount measured (A_m) of the constituents in Paeoniae Radix

Constituents	CZE					MEKC				
	Recovery (%)	Intra-day RSD (%)		Inter-day RSD (%)		Recovery (%)	Intra-day RSD (%)		Inter-day RSD (%)	
		t_m	A_m	t_m	A_m		t_m	A_m	t_m	A_m
AF						96.4	0.49	2.80	0.59	3.95
PF						93.1	0.70	1.85	0.70	3.14
BAF						102.1	0.54	2.27	0.67	3.41
PN	96.8	0.76	1.21	0.80	1.67	97.8	1.40	3.18	1.43	3.66
OPF	97.2	0.84	3.43	0.84	3.43	97.3	0.93	3.34	1.02	4.74
BA	95.1	0.53	1.46	1.22	1.87					
PG	95.4	1.05	2.54	1.37	3.18					
GA	94.3	0.95	3.88	0.98	3.92					

Suitable amounts (0.14–5.45 mg) of the eight constituents were added to a sample of Paeoniae Radix of known constituent content, the mixture was analysed using the proposed method. The recoveries of these compounds were 94.3–97.2% in CZE and 93.1–102.1% in MEKC. The reproducibilities (relative standard deviations) of each compound for six replicate injections are shown in Table 2.

The work has successfully demonstrated that by optimizing parameters such as borate concentration and pH value in CZE and surfactant and methanol concentration in MEKC, high resolution separations of the crude extracts of Paeoniae Radix samples can easily be achieved.

Acknowledgments

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References

[1] H.Y. Hsu, Y.P. Chen, S.J. Sheu, C.H. Hsu, C.C. Chen and H.C. Chang, Chinese Material Medica - A Concise Guide, Oriental Healing Arts Institute, Los Angeles 1985, p. 474.
[2] M. Kaneda, Y. Iitaka and S. Shibata, Tetrahedron, 28 (1972) 4309.
[3] K. Yamasaki, M. Kaneda and O. Tanaka, Tetrahedron Lett., 44 (1976) 3965.
[4] S. Hideyo, Shoyakugaku Zasshi, 38 (1984) 144.
[5] S. Shibata and M. Nakahara, Chem. Pharm. Bull., 11 (1963) 372.
[6] N. Aimi, M. Inaba, M. Watanabe and S. Shibata, Tetrahedron, 25 (1969) 1825.
[7] M. Nishioka, T. Yamagishi, G. Nonaka and I. Nishioka, Chem. Pharm. Bull., 28 (1980) 2850.
[8] K. Takagi and M. Harada, Yakugaku Zasshi, 89 (1969) 879, 887, 893, 899.
[9] K. Takechi and Y. Tanaka, Planta Med., 45 (1982) 252.
[10] C.S. Shim, I.S. Kim and S.Y. Paik, Koryo Taehakkyo Uikwa Taehak Chapchi, 17 (1980) 183; Chem. Abstr., 93 (1980) 107257m.
[11] I. Nishizawa, T. Yamagishi, T. Horikoshi and N. Homma, Chem. Pharm. Bull., 28 (1980) 2850.
[12] C. Jin, G. Xu, R. Jin, L. Xu and X. Cong, Dongguo Yaoke Daxue Xuebao, 20 (1989) 139; Chem. Abstr., 111 (1989) 160359n.
[13] S. Honda, K. Suzuki, M. Kataoka, A. Makino and K. Kakehi, J. Chromatogr., 515 (1990) 653.
[14] Y.M. Liu and S.J. Sheu, J. Chromatogr., 600 (1992) 370.
[15] Y.M. Liu and S.J. Sheu, J. Chromatogr., 623 (1992) 196.
[16] Y.M. Liu and S.J. Sheu, J. Chromatogr., 634 (1993) 329.
[17] Y.M. Liu and S.J. Sheu, J. Chromatogr., 637 (1993) 219.
[18] D.N. Heiger, in High Performance Capillary Electrophoresis - An Introduction, Hewlett–Packard Company, France 1992, p. 36.