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Optimized separation of pharmacologically active flavonoids from *Epimedium* species by capillary electrophoresis

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Abstract

Sixteen pharmacologically active flavonoids and one phenylethanoid glucoside isolated from *Epimedium* species were analyzed by capillary electrophoresis in different buffer systems of AMPSO, CAPS, phosphate and borate. The borate system proved to be the best. After optimization and studies of the effect of borate and SDS concentrations, organic modifier, voltage and effective capillary length on migration of the analytes, 14 flavonoids and one phenylethanoid glucoside were baseline separated within 20 min in the buffer system of 20 m*M* borate and 48 m*M* SDS containing 1 m*M* 1, 3-diaminopropane at pH 8.5 and at the detection wavelength of 254 nm. The two-marker (xanthene-9-carboxylic acid/*meso*-2,3-diphenylsuccinic acid) technique and the computer program based on MATLAB were applied to improve the repeatability of analysis and to confirm the reliability of identification between two successive compounds. The reliability of identification was enhanced up to 25-fold for the analytes when the migration indices were used with the two-marker technique.

Keywords: Buffer composition; *Epimedium* species; Flavonoids; Glucosides

1. Introduction

Flavonoids are plant constituents comprising one of the most numerous and widespread groups of natural products. They have a variety of biological effects such as antiviral, anti-allergic, anti-anginal, anti-inflammatory, antihepatoxic, antimicrobial, anti-ulcer, and spasmolytic. They are also inhibitory to a series of enzymes (e.g., hydrolylase, ATPases, cAMP phosphodiesterases, kinases, lipases and tranferases). There have been continuing interests in studying the natural flavonoids as potential new drugs [1]. Herba Epimedii from many species of the genus *Epimedium* (family Berberidaceae) has been a commonly used Chinese herbal medicine, and has proven to have efficacy against cardiovascular diseases and other chronic illness (infertility, impotence and senile functional diseases) for over 2000 years. Flavonoids are the major and active constituents from the genus. The flavonoid extracts from the species are pharmacologically active. They are effective on dilation of the coronary artery, inhibition of the platelet aggregation, and delayed formation of thrombi. They can also improve humoral and cellular immunity, increase synthesis of DNA and exert anti-aging effects [2]. Over 40 flavonoids have been isolated from the genus until now. Several methods such as pulse polarography, coulometric titration, fluorometry and

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liquid chromatography have been used to study the flavonoids from *Epimedium* species [3].

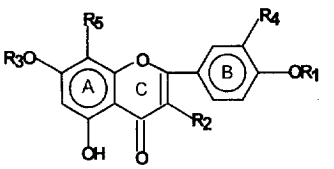
Capillary electrophoresis (CE) is important as an analytical separation technique because of its efficiency, flexibility and accuracy [4]. Micellar electrokinetic capillary chromatography (MEKC), a hybrid of electrophoresis and chromatography, is one of the most widely used CE modes. In MEKC, a surfactant is added to a buffer at a concentration above its critical micelle concentration (CMC). Micelles provide both ionic and hydrophobic sites of interaction. CE was, however, not applied to the separation of flavonoids until 1991 [5]. Morin et al. have investigated five flavonol glycosides differing in the types of sugar moiety in capillary zone electrophoresis (CZE) [6]. Seitz et al. have used capillary isotachophoresis for the determination of flavonoids and phenylcarboxylic acids [7]. Bjergegard and his coworkers have studied flavonoid glycosides by using cetyltrimethylammonium bromide (CTAB) or cholate in MEKC [8]. Furthermore, Liu et al. determined six flavonoids in *Scutellaria* by SDS–MEKC [9]. In addition, the separation of flavonoid aglycones have been discussed in several papers [10–14].

Until now, only dozens out of over 4000 flavonoids not from a particular taxonomic group (species, genus, family, or closely related group of families) have been studied with CE [5–14]. The separation of compounds from the same genus is of significance for qualitative and quantitative analysis and of importance for chemical taxonomy of the genus. In our study, all flavonoids and one phenylethanoid glucoside are isolated from the genus *Epimedium*. These compounds had not earlier been studied with CE or MEKC.

The repeatability of migration times in CZE and MEKC is usually at 1–2% level. This is mainly due to nonrepeatable electroosmotic flow velocity (v_{eo}) caused by the unstable surface conditions of the capillary wall and the changes in effective electric field strength (E_{eff}) [15]. The repeatability of CZE can be improved by making corrections for the changes in v_{eo} and E_{eff}. This can be done by introducing marker compounds with known electrophoretic mobility [16]. In MEKC, the net mobility of a compound is determined by its own electrophoretic mobility and its mobility while partitioned into a micelle. The electrophoretic mobility can be replaced with the indices of the marker compounds in order to find the total mobility of the markers in a micellar system [17].

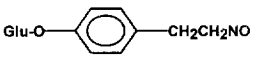
The compounds studied include 16 flavonoids and one phenylethanoid glucoside commonly isolated from *Epimedium* (Fig. 1). Flavonoids have structures based on 2-phenylbenzopyrone and differ in the degree and pattern of hydroxylation and glycosylation as well as in the type and position of sugar links. The characteristics of tested compounds include: (1) the glycosides and aglycones with different substituents; (2) the same aglycones differing in the degree of glycosylation; (3) the same number, link type and position of sugar moiety with different patterns of hydroxylation of aglycones; (4) the same number, link position of sugar moiety and aglycone with different types of sugars; (5) constituents with differences only in the terminal sugars.

The aim of present work was to study the effect of electrolytes, surfactant, organic modifier and some instrumental parameters on the migration behavior of the flavonoids and one phenylethanoid glucoside,



No	Name	R_1	R_2	R_3	R_4	R_5
2	luteolin-7-O-glucoside	H	H	glu	OH	H
3	8-methoxy-isoquercitrin	H	O-glu	H	OH	OCH_3
4	vitexin	H	H	H	H	C-glu
5	hyperin	H	O-gal	H	OH	H
6	quercitrin	H	O-rha	H	OH	H
7	quercetin	H	OH	H	OH	H
8	kaempferol-3-O-rha	H	O-rha	H	H	H
9	luteolin	H	H	H	OH	H
10	tricin	H	H	H	OCH_3	H (5'-OCH_3)
11	desmethylicaritin	H	OH	H	H	prenyl
12	icariin II	CH_3	O-rha	H	H	prenyl
13	2''-O-rhamnosylicariside I	CH_3	O-rha-rha	H	H	prenyl
14	icariin I	CH_3	OH	glu	H	prenyl
15	icariin	CH_3	O-rha	glu	H	prenyl
16	epimedin C	CH_3	O-rha-rha	glu	H	prenyl
17	epimedin B	CH_3	O-rha-xyl	glu	H	prenyl

glu: glucose; rha: rhamnose; xyl: xylose; gal: galactose



1 thalictoside

Fig. 1. Structures of the investigated compounds.

and to develop an effective and rapid screening method for them. The repeatability of the optimized separation analysis was tested and the reliability of identification was confirmed by applying the two-marker technique.

2. Experimental

2.1. Apparatus

The separation was performed on a Waters Quanta 4000 capillary electrophoresis system (Millipore, Waters Chromatography Division, Milford, MA, USA) using 0.050 mm I.D.×360 mm O.D. fused-silica capillary (Composite Metal Services, Worcestershire, UK).

The data were collected by an Hewlett-Packed model 3392A integrator (Avondale, PA, USA). Migration indices were computed with in-house programs developed for MATLAB [16]. The data was analyzed with Microsoft Excel Ver. 5.0.

The pH of the buffers was adjusted with a Jenway 3030 pH meter connected to a Jenway electrode (Jenway, Felsted, UK) containing 4 *M* KCl in saturated AgCl. The electrode system was calibrated with potassium hydrogenphthalate (pH 4.00) and borate/sodium hydroxide solutions (pH 11.00). A Water-I system from Gelman Sciences (Ann Arbor, MI, USA) was used for ion-exchange of the distilled water.

2.2. Materials and reagents

The flavonoids and the phenylethanoid glucoside were isolated from the aerial parts of *Epimedium* species by liquid chromatography (LC). Their structures have been identified by spectroscopic methods (UV, IR, FAB-MS, MS–MS, ^{1}H, ^{13}C-NMR, ^{1}H-^{1}H COSY, DEPT, HMQC, HMBC and TOCSY) [18].

All chemicals were of analytical-reagent grade: sodium dodecyl sulfate (SDS), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), [3-(1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO), disodium hydrogenphosphate dihydrate and sodium dihydrogen phosphate dihydrate (phosphate) from Sigma (St. Louis, MO, USA); disodium tetraborate decahydrate (borate) from E. Merck (Darmstadt, Germany); methanol, 2-propanol and acetonitrile from Rathburn Chemicals (Walkerburn, UK); xanthene-9-carboxylic acid from EGA-Chemie (Steinheim, Germany); and *meso*-2,3-diphenylsuccinic acid from TCI (Japan). Water used for dilution and buffer solutions was distilled, deionized and filtered.

2.3. Procedure

Buffer solutions were prepared from AMPSO, CAPS, phosphate and borate, and their pH was adjusted to 10.5. The pH of buffer solutions from borate containing SDS was adjusted to 8.5. All the buffer solutions were filtered through 0.45 μm membrane filters (Millipore, Ireland) and degassed before use.

New capillary tubes were purged with 0.1 *M* NaOH, water and buffer successively. The capillaries were conditioned daily by washing with 0.1 *M* NaOH, water and buffer successively for 3×5 min. Between consecutive analyses, the capillary was purged for 2 min with the buffer solution.

Analytes were dissolved in methanol (1000 ppm) and further diluted with 30% aqueous methanol to obtain reference solutions (20–200 μg/ml). Stock solutions of 1000 ppm of each marker (xanthene-9-carboxylic and *meso*-2,3-diphenylsuccinic acid were prepared in methanol. All solutions were filtered through 0.45 μm pore size membranes (Gelman Sciences). 20 μl of xanthene-9-carboxylic acid and 150 μl of *meso*-2,3-diphenylsuccinic acid were then added to tubes containing 400 μl of sample solutions.

Samples were introduced into a capillary from its anodic end by hydrostatic mode for 8 s. The capillary of total length was varied from 53 to 73 cm, effective length from 45 to 65 cm. The running voltage, which varied with the application from 16 to 25 kV, was kept constant once selected. Detection was performed at the wavelength of 254 nm. All experiments were carried out at ambient temperature.

2.4. Two-marker technique

In the two-marker technique, the migration index of one marker with the shorter migration times is set

to 1000 (Ind_1), then Ind_2 can be calculated according to Eq. (1) [17].

$$Ind_2 = Ind_1(t_{eo}/t_2 - 1)/(t_{eo}/t_1 - 1) \quad (1)$$

where Ind_1 and Ind_2 are the migration indices of markers 1 and 2, t_1 and t_2 are their respective migration time, and t_{eo} is the electroosmotic migration time. The migration indices of the analytes (Ind_x) can then be calculated by (2)

$$Ind_x = [t_1 t_2(Ind_1 - Ind_2) - t_x(Ind_1 t_1 - Ind_2 t_2)]/t_x(t_2 - t_1) \quad (2)$$

3. Results and discussion

3.1. Choice of electrolyte solutions

Flavonoid compounds are weak acids with ionization constants (pK_a) from 9–12 due to the presence of phenolic hydroxyl group; their apparent charge depends on their pK_a values and pH of the sample and the buffer. Our studies showed that at neutral pH, the flavonoids studied coeluted with different phosphate buffer concentrations (30–100 m*M*). Due to the phenolic nature of the flavonoids, an alkaline buffer system (AMPSO, CAPS, phosphate and borate) was chosen. Electrolyte solutions were tested at pH 10.5 with concentrations of 20, 50 and 80 m*M*. Even though AMPSO and CAPS electrolytes generated lower electric current inside the capillary, borate buffer proved to be the best buffer system. Nine from 17 compounds could be separated by CZE in the borate concentration of 20 m*M*. In addition to the stabilization of the pH, the borate ions most probably form complexes with flavonoid aglycones or/and saccharides enhancing the selectivity of separation.

3.2. Further optimization of the complexing conditions for better separation

Optimization of the complexing conditions was carried out by investigating the effect of SDS, borate and organic modifiers. Furthermore, the influence of effective capillary length and applied voltage on resolution of the analytes was studied.

3.2.1. Effect of SDS

According to the literature, the CMC of SDS is 8.2 m*M* in pure water at 25°C. Electrolyte solution seems to have an important influence on CMC of SDS; e.g., it is only 2.9 m*M* in borate–phosphate buffer (25°C) [19].

Effect of SDS concentrations ranging from 0 to 54 m*M* in 20 m*M* borate solution (pH 8.5) on the separation efficiency was studied (Fig. 2). The separation efficiency was enhanced with increasing SDS concentrations in the electrolyte solution while the migration times of the analytes were increased.

As low SDS concentration as 3 m*M* in the electrolyte solution improved the resolution. According to our studies the CMC of SDS in the 20 m*M* borate buffer (pH 8.5) at ambient temperature seems to be around 4.5 m*M* (Fig. 2). However, higher SDS concentrations were needed to improve the separation. At 12 m*M* SDS ten compounds (including 15, 16 and 17) were separated with poor resolution. When the concentration increased up to 20 m*M*, the resolution was further improved. The resolution of aglycones (7, 9, 10) was gradually improved with further increasing concentrations of SDS while that of di- or trisaccharidic flavonoid glycosides (15, 16, 17) was not improved. The aglycones (7, 9) migrated with slower speed than their corresponding glyco-

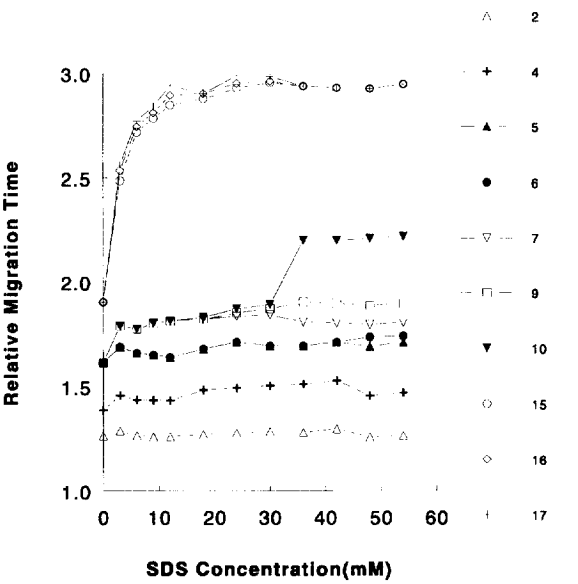


Fig. 2. Influence of SDS concentration on relative migration time. Conditions: 20 m*M* borate, pH 8.5 and 20 kV. Numbers are the same as Fig. 1.

sides (2, 6) due to glycosylation which makes these flavonoids more hydrophilic and water soluble. Most of the investigated compounds were separated at the SDS concentration of 48 m*M*.

3.2.2. Effect of borate concentrations in MEKC

Optimization with six borate concentrations from 10 to 36 m*M* in the fixed SDS concentration (48 m*M*, pH 8.5) was investigated. The t_{eo} value (methanol) and the migration times of the analytes increased as borate concentration increased. The borate concentration influenced mostly the structures with *cis*-diol groups on flavonoid skeleton and /or saccharide moieties. Analytes 2, 5 and 6 with vinicial hydroxyl groups of B-rings migrated faster than analyte 8 with one hydroxyl group, probably because the presence of vinicial hydroxyl groups of B-rings (Fig. 1) is more favorable for their complexation with borate and thus greater affinity for the aqueous phase than micellar phase.

3.2.3. Effect of organic modifiers in MEKC

2-propanol and acetonitrile from 5 to 15% (v/v) were first used to further optimize the buffer system. The resolution of analytes was improved, but the migration time became longer and the peak shapes were widened.

However, very low concentration addition (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 m*M*) of 1,3-diaminopropane showed more ideal result. The best separation was achieved with 20 m*M* borate and 48 m*M* SDS containing 1 m*M* 1,3-diaminopropane (Fig. 3). 1,3-diaminopropane had more pronounced effect on the migration times of analyte 11, 12, 13 and 15 than on those of other compounds.

3.2.4. Effect of effective instrumental parameters in MEKC

Changing the effective capillary lengths from 45 to 65 cm and applied voltages from 16 to 25 kV showed that the length should be 50 cm and the applied voltage below 25 kV under the analytical conditions, in order to avoid excessive current inside the capillary.

The final separation was obtained by using 20 m*M* borate and 48 m*M* SDS containing 1 m*M* 1,3-diaminopropane (pH 8.5), with an effective capillary length of 50 cm, and applied voltage at 20 kV. 14 flavonoids and one phenylethanoid glucoside were separated within 20 min (Fig. 4).

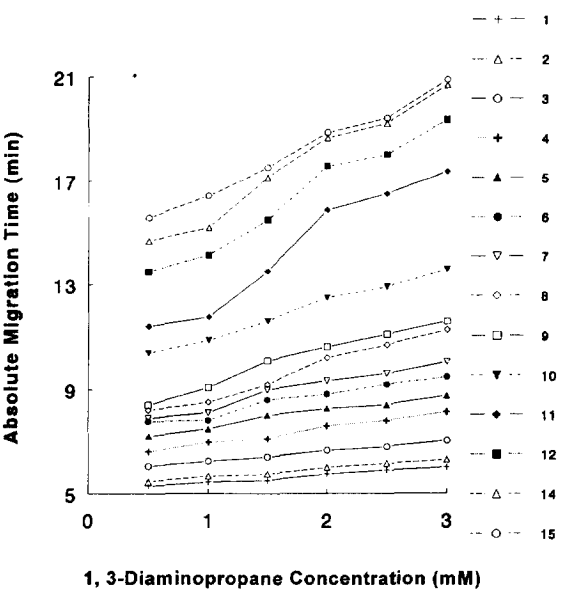


Fig. 3. Effect of 1, 3-diaminopropane on migration time. Conditions: 20 m*M* borate, 48 m*M* SDS, pH 8.5 and 20 kV. Numbers are the same as Fig. 1.

3.3. Application of the two-marker technique

3.3.1. Repeatability of analysis

The repeatability was tested by applying the two-marker technique developed for CZE [16,17]. The two markers, xanthene-9-carboxylic acid and *meso*-2,3-diphenylsuccinic acid were used for the calculation of repeatability (Fig. 4). The migration indices were determined according to Eqs. (1) and (2). As seen in Table 1 the repeatability of nine replicates with migration indices was superior to that calculated by using absolute migration times.

3.3.2. Reliability of the identification

The coefficient for identification Q_{id} was introduced to express the reliability of the identification [16]

$$Q_{id} = (x_2 - x_1)/(\sigma_2 + \sigma_1) \quad (3)$$

where Q_{id} is indicative of the reliability of identification between two successive compounds. Eq. (3) is a direct application of the resolution equation in chromatography. If Q_{id} value exceeds two, the identification between two compounds is considered reliable. x_1 and x_2 are the absolute migration times or

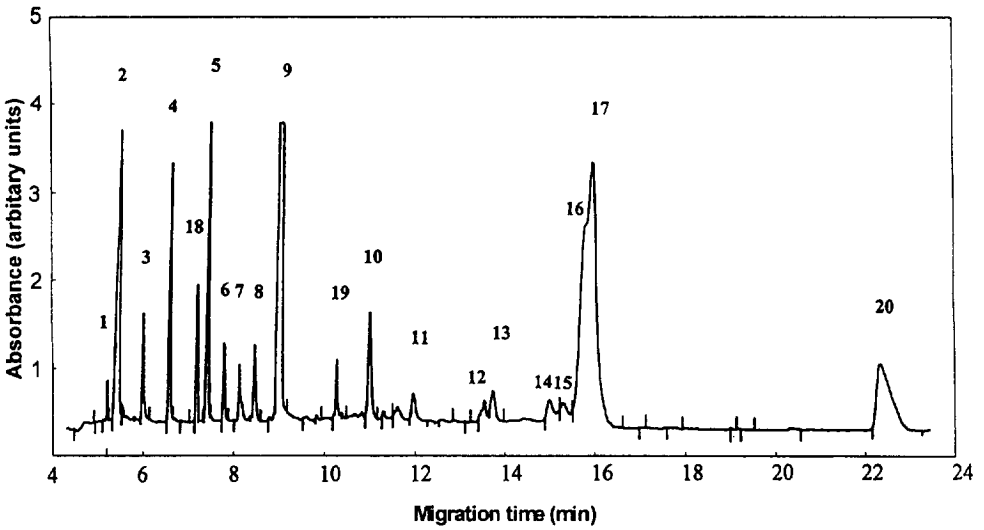


Fig. 4. The electropherogram of the analytes. Conditions: 20 m*M* borate, 48 m*M* SDS, 1 m*M* 1,3-diaminopropane, pH 8.5 and 20 kV with an effective capillary length of 50 cm. Numbers are the same as Fig. 1; 18= xanthene-9-carboxylic acid; 19= *meso*-2,3-diphenylsuccinic acid; 20= *o*-phthalic acid; the migration time of methanol= 4.01 min.

migration indices, and σ_1 and σ_2 are their standard deviations. Introduction of the migration indices resulted in reliable identification for the analyte pairs (except one analyte pair) while the migration times failed to give rise to reliable identification for five analyte pairs. The reliability of identification with the migration indices was 25 times better than that with absolute migration times (Table 2).

4. Conclusions

Effective separation of 16 flavonoids and one phenylethanoid glucoside isolated from *Epimedium* by MEKC required the careful optimization of electrolytes, surfactants, organic modifiers and instrumental parameters. According to our studies, MEKC with the buffer system of borate and SDS

Table 1
Repeatability of analyses relying on absolute migration times and on migration indices of the analytes (nine replicates)

Analyte	Absolute migration time (min)			Migration index		
	t_{ab} (Mean)	S.D.	R.S.D. (%)	Mean Index	S.D.	R.S.D. (%)
1	5.36	0.06	1.03	502.5	2.20	0.44
2	5.61	0.06	1.01	584.5	3.80	0.65
3	6.14	0.07	1.15	738.1	0.54	0.07
4	6.71	0.08	1.15	877.1	1.18	0.13
5	7.53	0.09	1.14	1040.2	1.77	0.17
6	7.89	0.08	1.18	1100.7	1.93	0.18
7	8.24	0.09	1.13	1152.8	2.46	0.21
8	8.56	0.10	1.11	1198.6	2.80	0.23
9	9.09	0.09	0.98	1265.4	2.36	0.19
10	11.06	0.16	1.46	1464.2	5.16	0.35
11	12.00	0.20	1.65	1536.3	3.59	0.23
12	13.31	0.18	1.35	1604.9	2.16	0.13
13	13.65	0.15	1.10	1635.2	2.15	0.13
14	15.02	0.06	0.40	1696.9	1.61	0.09
15	15.47	0.04	0.27	1720.8	2.33	0.14
16	15.85	0.21	1.34	1736.9	8.94	0.51

Table 2
Values of Coefficient of Identification (Q_{id}) obtained for successive peak pairs

Analyte pair	Absolute migration time Q_{id} (t_{ab})	Migration index Q_{id} (ind)	Ratio Q_{id} (ind)/Q_{id} (t_{ab})
1/2	2.2	13.7	6
2/3	4.2	35.4	9
3/4	3.9	81.2	21
4/5	5.1	55.3	11
5/6	2.1	16.3	8
6/7	1.8	11.9	6
7/8	1.7	8.7	5
8/9	2.9	12.9	4
9/10	7.9	26.4	3
10/11	2.6	8.2	3
11/12	3.5	12.0	4
12/13	1.1	7.0	7
13/14	2.2	16.4	7
14/15	0.2	6.1	25
15/16	0.6	1.4	2

containing a low concentration of 1,3-diaminopropane proved to be a very effective method for analysis of flavonoids from the genus. 14 flavonoids and one phenylethanoid glucoside were accurately identified by MEKC within 20 min in a single run. The two-marker technique improved the repeatability of the analysis and reliability of the identification. The MEKC system developed has the potential for a rapid separation and further specific determination of flavonoids from the traditional Chinese drugs of Herba Epimedii and taxonomic studies of the genus (*Epimedium*).

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