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Separation and simultaneous determination of rutin, puerarin, daidzein, esculin and esculetin in medicinal preparations by non-aqueous capillary

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Abstract

A simple method for the simultaneous determination of five bioactive components (rutin, puerarin, daidzein esculin and esculetin) in traditional medicinal preparations by non-aqueous capillary electrophoresis with UV detection has been developed for the first time. A running buffer composed of 15% acetonitrile, 2.5% acetic acid and 90 mM sodium cholate in methanol was found to be the most suitable for this separation. The limits of detection for five analytes were over the range of 0.050–1.216 $\mu g\,ml^{-1}$. The relative standard deviations (R.S.Ds.) of the migration times and the peak areas of the analytes were in the range of 1.3–2.9% and 2.2–2.7% (intraday), 1.7–1.9% and 2.8–3.6% (interday), respectively. In the tested concentration range, linear relationships (correlation coefficients: 0.9974 for rutin, 0.9976 for puerarin, 0.9981 for daidzein, 0.9972 for esculin and 0.9929 for esculetin) between peak areas and concentrations of the analytes were obtained. This method has been successfully applied to simultaneous determination of the five bioactive components with recoveries over the range of 89.4–107.4%.

Keywords: Non-aqueous capillary electrophoresis; Rutin; Puerarin; Daizdein; Esculin; Esculetin; Medicinal preparations

1. Introduction

Traditional Chinese medicinal herbs are a rich natural resource and have been extensively used to prevent and cure many diseases that inflicted humans for thousands of years in China. Traditional Chinese medicine has become increasingly popular in recent years owing to the advantages of low toxicity and rare complications. However, because a great number of these medicines may show a complicated profile of constituents, efforts to develop a simpler and more facile analytical method that can assay as many bioactive components as possible are quite necessary, especially for the control of quality.

There are several kinds of Chinese medicinal preparations used to cure the common cold, influenza and inflammation, such as Xiaoke pill, Yufengningxin pill and Geqin-tang (composed of *Radix puerariae*, *Cortex fraxini* and *Fructus sophorae*). Puerarin and daidzein are the major active components of *Radix puerariae*. Esculin and esculetin are the two of the most important bioactive components in *Cortex fraxini*. Rutin is one of important bioactive components in *Fructus sophora* and presents in *Radix puerariae* [1]. Puerarin, daidzein and rutin have many important physiological activities such as antiproliferative effects on human cancer cell lines, inhibiting alcohol dehydrogenase and xanthine oxidase, anti-oxidant activity, tumor growth-inhibitory activity in various cancer cell lines in vitro [2–4]. Esculin and esculetin have multiple biological activities including inhibition of xanthine oxidase activity, anti-oxidant activity, antitumor activity, and inhibitory effect on the growth of human breast cancer cells [5,6]. Therefore, it is urgently needed to establish a simple and effective method for the quantitative analysis of these components.

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At present, many analytical methods have been used to analyze these compounds, including thin-layer chromatography (TLC) [7–10], high-performance liquid chromatography (HPLC) [11–18]. However, TLC lacks quantitative precision and separation power. Although HPLC possesses higher accuracy, it has lower efficiency and a longer analysis time. In recent years, capillary electrophoresis is introduced into the medical analysis for its high separation efficiency, low sample consumption, short analysis time, lower operating cost and relatively simple instrumentation. Chen et al. determined rutin in plants by CE [19]. Chen et al. determined puerarin, daidzein, rutin in *Pueraria lobata* by CE [20], Peng et al. determined daidzein in soy products by CE, with 20 min [21], Cao et al. determined puerarin, daidzein in *Radix puerariae* by CE [22], Wang et al. determined rutin in Chinese herb medicines and human urine by CE [23], Jiang et al. determined rutin in cigarettes by CE [24], Huang and Hsieh determined puerarin, daidzein in Ge-gen-tang by CE [25], Wang et al. determined puerarin, daidzein in medicinal preparations by CE [26], Zhang et al. determined aesculin and aesculetin in *Cortex fraxini* by CE [27] and You et al. determined esculin and esculetin in ash bark by CE [28]. However, the method of simultaneous determination of rutin, puerarin, daidzein, esculin and esculetin has not been reported.

Non-aqueous capillary electrophoresis (NACE) offers a number of advantages over CE methods that use aqueous or mixed media, such as the increased solubility of analytes, lower Joule heating and the reduced interaction of hydrophobic analytes with the negative charge capillary wall. These advantages stem from the wide array of physical and chemical properties that organic solvents possess. NACE has been found to be a good alternative for the analysis of pharmaceuticals and their metabolites that are water-insoluble and difficult to be separated in aqueous media [29–35].

According to our knowledge, there has been no report on the determination of the five bioactive components by NACE or the simultaneous determination of the five bioactive ingredients in their medicinal preparations. Considering the poorly water-soluble of the investigated substances, the aim of this paper is to develop a non-aqueous capillary electrophoresis method for the simultaneous determination of these in complicated medicinal preparations for the first time, and good results were obtained.

2. Experimental

2.1. Apparatus

All experiments were performed on a P/ACE^{TM} MDQ system (Beckman Coulter Instrument, Fullerton, CA, USA) equipped with UV detector. The system was controlled by 32 Karat^{TM} software (version 7.0). The separation was carried out on a 50.2 cm (40 cm from inlet to the detector) × 75 μm I.D. fused-silica capillary (Yongnian Photoconductive Fiber Factory, Hebei, China). Prior to its first use, the capillary were preconditioned by rinsing with 1.0 M HCl for 10 min, water for 5 min, 0.50 M NaOH for 10 min, methanol for 5 min, and acetonitrile for 5 min. Each day, the capillary were conditioned by flushing with methanol for 5 min, followed by acetonitrile for 5 min. Between two runs, the capillary was flushed with the separation buffer for 5 min. When not in use, the capillary was washed with methanol and water for 5 min each, and then dry-stored. Samples were injected by applying a pressure of 0.3 psi for 2 s. The detection wavelength was set at 254 nm.

2.2. Materials and reagents

Rutin (R), puerarin (P), daidzein (D), esculin (EL) and esculetin (ET) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China. Acetonitrile and acetic acid were purchased from Tianjin First Chemical Factory. Methanol was obtained from Shanghai Zhenxing First chemical Factory. Sodium cholate (SC) was purchased from Serva Feinbiochemica (Heidelberg, New York). *Radix puerariae*, Geqin-tang, Yufengningxin pill and Xiaoke pill, were purchased from Renren pharmaceutical store, Jiaozuo, China. All reagents were of analytical grade.

Stock solutions of rutin (2400.0 μg ml^{-1}), puerarin (1000.0 μg ml^{-1}), daidzein (700.00 μg ml^{-1}), esculin (2800.0 μg ml^{-1}) and esculetin (800.00 μg ml^{-1}) were prepared in methanol. Less concentrated standard solutions were prepared by diluting the stock solutions with methanol.

The running buffers were prepared by mixing 200.0 mM sodium cholate (in methanol), acetic acid, acetonitrile and methanol.

2.3. Sample preparation

The samples were finely powdered and grinded, and then 1.0124 g for *Radix puerariae*, 0.9360 g for Geqin-tang, 1.0158 g for Yufengningxin pill and 1.0079 g for Xiaoke pill were accurately weighed, respectively. The weighed samples were extracted with 15 ml of methanol for 30 min in an ultrasonic bath. The extracted solution was then filtered through filter papers. The extraction procedure was repeated three times. The total extracted solution was concentrated to near-dryness, and methanol was added to dissolve the residue to a volume of 10 ml for *Radix puerariae*, Yufengningxin pill and Xiaoke pill, 25 ml for Geqin-tang. Finally, 2-fold-diluted (Xiaoke pill), 4-fold-diluted (*Radix puerariae* and Geqin-tang) and 16-fold-diluted (Yufengningxin pill) with methanol before use. All the buffer, standard and sample solutions were filtered through 0.45 μm membrane filters before been injected into the capillary electrophoresis system before analysis by NACE.

The peak identification was performed by adding standards to the sample solutions.

3. Results and discussion

3.1. Separation conditions

Since rutin, puerarin, daidzein, esculin and esculetin were hydrophobic compounds, their structures were showed in Fig. 1, non-aqueous capillary electrophoresis system was highly advantageous to their separation. They had hydroxyl groups in their molecules and could form negative complex ions by combining their hydroxyls with sodium cholate ions under basic sodium cholate conditions. Because they had different ionization abilities, they had different charge–mass ratios and could be separated for their different electrophoretic mobility under high electric field. The influence of several experiment parameters, such as the effects of the acetontrile percentage, acetic acid percentage, sodium cholate concentration, applied voltage and capillary temperature on the separation, were systematically investigated.

The buffer concentration played a prominent role in electrophoretic separation. Because the buffer concentration influenced the viscosity of the solution, the diffusion coefficient of analytes and the ξ-potential of the inner surface of capillary, it affected the resolution and migration time. The effects of the concentration of sodium cholate running buffers (from 70 to 110 mM) on the separation were investigated, and the results were shown in Fig. 2. When the sodium cholate concentration was increased, the electro-osmotic flow reduced and the migration time prolonged. In this experiment, it was also found that resolutions of all analytes increased with increasing concentration of sodium cholate. Therefore, 90 mM sodium cholate was selected for subsequent experiments in considering analytical time and resolution.

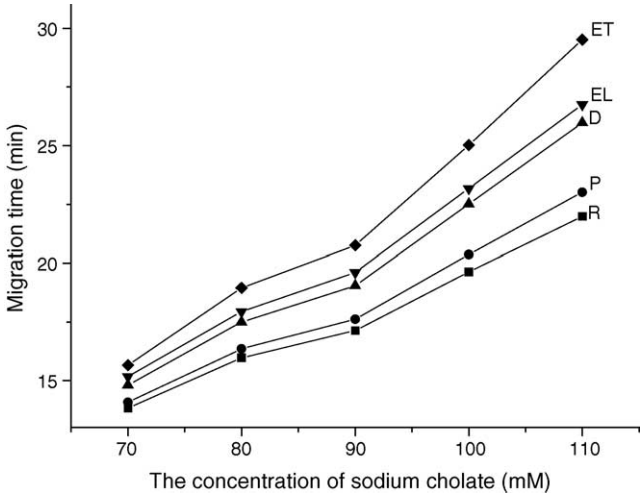

Fig. 2. Effect of sodium cholate concentration on the separation. R, rutin; P, puerarin; D, daidzein; EL, esculin; and ET, esculetin. Buffer: containing 3.5% acetic acid and 20% acetonitrile with different sodium cholate concentration (from 70 to 110 mM) in methanol; capillary: uncoated fused-silica capillary 50.2 cm (40 cm injector to detector) × 75 μm i.d.; UV detection wavelength: 254 nm; applied voltage: 25 kV; capillary temperature: 289 K.

The acidity of the buffer was also a governing factor in separation of ionizable analytes because it affected the ξ-potential and the overall charge of the analytes, which

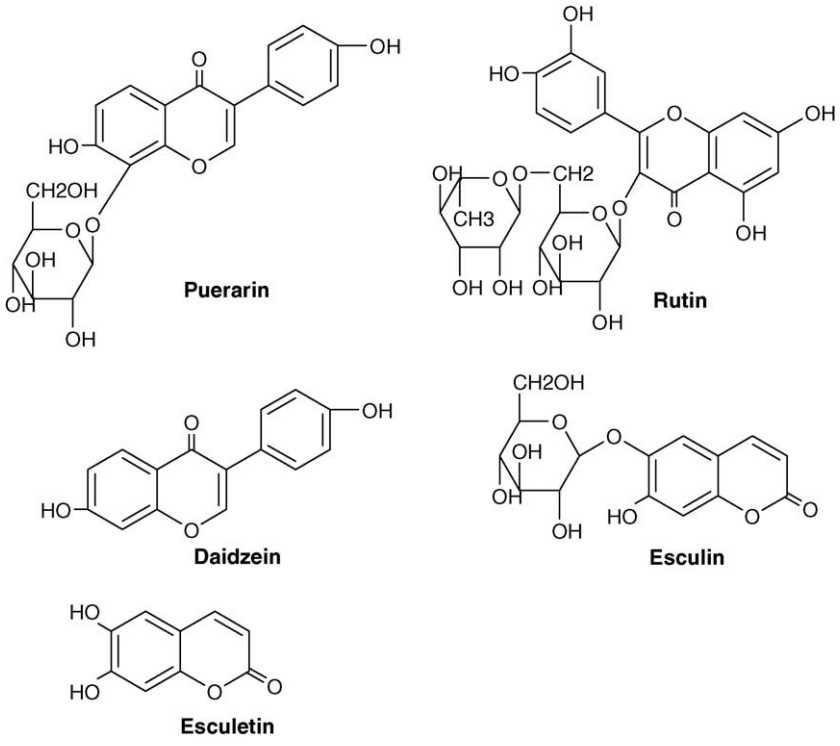

Fig. 1. Structure of the five analytes.

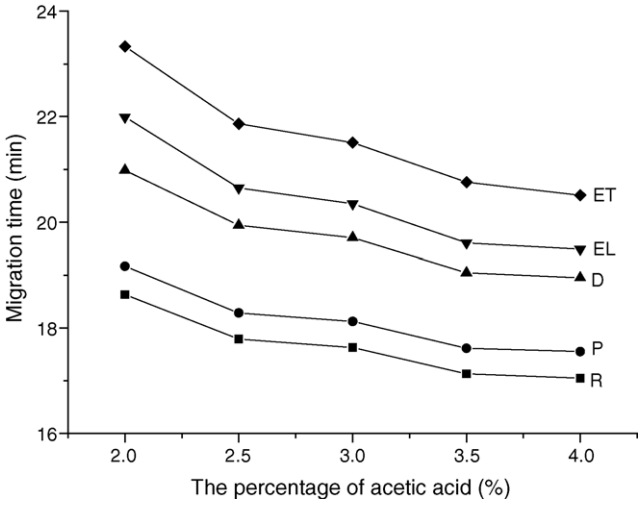

Fig. 3. Effect of the acidity on the separation. Buffer: 90 mM sodium cholate, 20% acetonitrile, different percentage of acetic acid (from 2 to 4%) in methanol. Other conditions see Fig. 2.

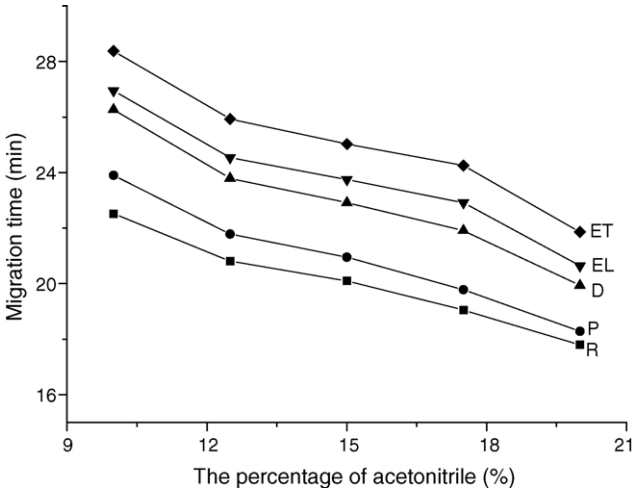

Fig. 4. Effect of acetonitrile percentage in methanol on the separation. Buffer: 90 mM sodium cholate, 2.5% acetic acid, different percentage of acetonitrile (from 10 to 20%) in methanol. Other conditions see Fig. 2.

determined the migration time and the separation of the analytes. The acidity of buffer was adjusted by acetic acid. Running buffers composed of 20% acetonitrile and 90 mM sodium cholate with different percentages of acetic acid ranging from 2 to 4% were prepared to evaluate the influence on the analytes migration behavior. Fig. 3 indicate that the migration times of the analytes decreased with increasing the content of acetic acid in the running buffers. In the experiment, it was also found that rutin and puerarin could not be achieved at baseline separation when the content of acetic acid is over 3.5%. Therefore, 2.5% acetic acid was chosen as the running buffer in considering according to the experimental results.

Fig. 4 summarized the effects of acetonitrile percentage in methanol (from 10 to 20%) containing 90 mM sodium cholate and 2.5% acetic acid on the analytes migration behavior. The migration times of the analytes decreased with the increasing acetonitrile, but the resolutions between adjacent peaks also decreased when acetonitrile percentage increased from 10 to 20%. When acetonitrile percentage in methanol was over 20%, the peaks of puerarin and daidzein were partly overlapped. Therefore, 15% acetonitrile with 90 mM sodium cholate and 2.5% acetic acid was selected as the running buffer in considering resolutions and analysis time.

Table 2
Precision for the migration time and peak area

Analyte	Migration time R.S.D.% ($n = 5$)		Peak area R.S.D.% ($n = 5$)	
	Intraday	Interday	Intraday	Interday
Rutin	1.5	1.7	2.6	2.8
Puerarin	1.4	1.5	2.7	3.2
Daidzein	2.9	3.5	2.6	2.9
Esculin	1.3	1.8	2.2	2.1
Esculetin	1.6	1.9	2.5	3.6

The effect of the applied voltage on the separation was investigated in the experiment. Increasing of the voltage shortened the migration times of all analytes, but it increased the baseline noise and decreased detection limits. Moreover, higher separation voltage generated more Joule heat that led to the decrease of separation efficiency. When the separation voltage was lower, peak broadening was found. Based on experiments, 27.5 kV was chosen as the optimum applied voltage to accomplish a good compromise.

The effect of capillary temperature on the separation was also studied in the experiment. It was found that the analysis time decreased when the temperature increased. Moreover, when the temperature was at 298 K, the better separation was achieved. This was due to the fact that the increase in

Table 1
The regression data, LOD and LOQ

Compound	Regression equation[a]	Correlation coefficient	Linear range ($\mu g\,ml^{-1}$)	LOD[b] ($\mu g\,ml^{-1}$)	LOQ[c] ($\mu g\,ml^{-1}$)
Rutin	$Y = -57591.4 + 849.7X$	0.9974	120.0–480.0	0.977	3.37
Puerarin	$Y = -70168.2 + 1987.0X$	0.9976	50.00–200.0	0.121	0.521
Daidzein	$Y = -78845.8 + 2905.5X$	0.9981	35.00–140.0	0.050	0.195
Esculin	$Y = -30431.9 + 261.0X$	0.9972	140.0–560.0	1.216	4.094
Esculetin	$Y = -32499.7 + 744.3X$	0.9929	40.00–160.0	0.117	0.424

[a] Y and X were the peak area and concentration ($\mu g\,ml^{-1}$) of the analytes, respectively.
[b] The limit of detection (LOD) was obtained based on the signal-to-noise ration of 3.
[c] The Limit of quantitation (LOQ) was obtained based on the signal-to-noise ration of 10.

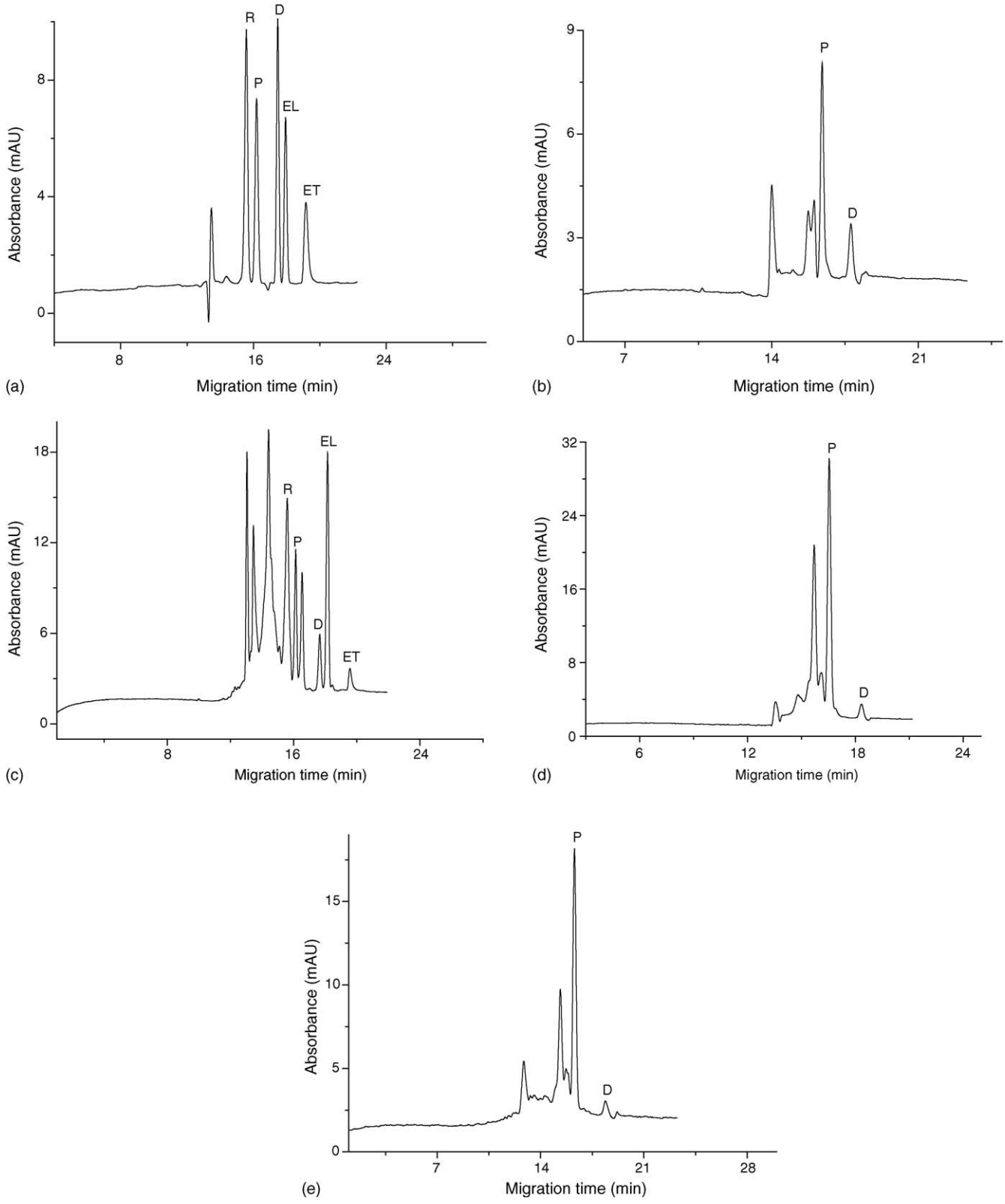

Fig. 5. Electropherogram of the standard mixture and the real samples. (a) The standard mixture; (b) *Puerariae radix*; (c) Geqin-tang; (d) Yufengningxin pill; (e) Xiaoke pill. Peaks: R, rutin; P, puerarin; D, daidzein; EL, esculin; ET, esculetin. Buffer: 90 mM sodium cholate, 2.5% acetic acid, 15% acetonitrile in methanol. Applied voltage: 27.5 kV. Capillary temperature: 298 K. Detection wavelength: 254 nm. Peaks were identified by standard addition method. Other conditions see Fig. 2.

Table 3
The determined results and average recoveries of the five analytes in samples ($n = 3$)

Sample	Compound	Content (mg g^{-1})	Added amount (μg ml^{-1})	Recovery (%)
Radix perariae	Rutin	ND[a]	300, 200, 150	106.3
	Puerarin	2.293 (2.8)[b]	150, 100, 75	96.3
	Daidzein	1.301 (2.4)	100, 75, 50	89.4
	Esculin	ND[a]	300, 200, 150	103.5
	Esculetin	ND[a]	150, 100, 75	103.1
Geqin-tang	Rutin	27.42 (3.1)	400, 300, 200	107.1
	Puerarin	7.761 (2.8)	150, 100, 75	95.4
	Daidzein	4.136 (2.5)	100, 75, 50	93.4
	Esculin	56.29 (2.6)	400, 300, 200	103.2
	Esculetin	6.665 (2.8)	150, 100, 75	103.2
Yufengningxin pill	Rutin	ND[a]	300, 200, 150	104.2
	Puerarin	27.62 (3.2)	150, 100, 75	96.4
	Daidzein	6.051 (3.2)	100, 75, 50	92.2
	Esculin	ND[a]	400, 300, 200	106.3
	Esculetin	ND[a]	150, 100, 75	103.4
Xiaoke pill	Rutin	ND[a]	300, 200, 100	107.3
	Puerarin	2.506 (2.9)	150, 100, 75	96.2
	Daidzein	0.761 (2.5)	100, 75, 50	92.1
	Esculin	ND[a]	400, 300, 200	106.6
	Esculetin	ND[a]	150, 100, 75	107.4

[a] ND not detected.
[b] The data in parentheses are R.S.D. (%).

temperature decreased the viscosity of the medium, and therefore decreased the migration time. Therefore, 298 K was selected as the optimum condition.

According to the experimental results above, the optimum separation was obtained with a running buffer containing 15% acetonitrile, 2.5% acetic acid and 90 mM sodium cholate in methanol at 27.5 kV and 298 K. A typical electropherogram obtained under the optimum separation condition for a standard mixture solution was shown in Fig. 5(a).

3.2. Method validation

The linear relationships between the concentrations of the analytes and the corresponding peak areas were investigated under the optimum separation condition. The results obtained were summarized in Table 1. The calibration curves exhibit good linear relationships over the tested concentration range of 120.0–480.0 μg ml^{-1} for rutin, 50.00–200.0 μg ml^{-1} for puerarin, 35.00–140.0 μg ml^{-1} for daidzein, 140.0–560.0 μg ml^{-1} for esculin and 40.00–160.0 μg ml^{-1} for esculetin, respectively.

The limit of detection (LOD) was considered as the minimum analytes concentration yielding a signal-to-noise ration equal to 3. The limit of quantification (LOQ) was obtained based on the signal-to-noise ration of 10. The determined results are listed in Table 1.

The precision and accuracy of the proposed method were determined by the measure of repeatability (intraday) and intermediate precision (interday). Repeatability was evaluated by relative standard derivation (R.S.D.) of replicate experiments ($n = 5$) of five analytes. The intermediate precision was assessed by measuring the R.S.D. of replicate experiments ($n = 5$) carried out at different days. In replicate experiments, the concentrations of five analytes were 170.0 μg ml^{-1} for rutin, 68.0 μg ml^{-1} for puerarin, 52.7 μg ml^{-1} for daidzein and 308.0 μg ml^{-1} for esculin and 89.0 μg ml^{-1} for esculetin, respectively. The results are summarized in Table 2.

3.3. Sample analysis

The new non-aqueous capillary electrophoresis method under the optimum separation condition was used to analyze rutin, puerarin, daidzein, esculin and esculetin in four practical samples according to the procedure in Section 2. The electropherograms of the four practical samples are shown in Fig. 5(b)–(e) and the resulting contents are shown in Table 3. The average recoveries of the method were determined with the standard addition method for the five analytes in the four sample solutions and the results are also given in Table 3. According to Table 3, satisfying results were obtained when the new method applied to the analysis of four actual samples. It meant that the new method was practical for the simultaneous determination of rutin, puerarin, daidzein, esculin and esculetin in complicated medicinal preparations.

4. Conclusion

The experimental results demonstrated that the new NACE method was practical for the simultaneous determination of rutin, puerarin, daidzein, esculin and esculetin in complicated Chinese medicinal preparations. The method exhibited many merits such as low sample volume, simultaneous analysis of the five bioactive components in the complicated medicinal preparations and high reproducibility. The new NACE method could be successfully used to the quality control of

complicated medicinal preparations containing rutin, puerarin, daidzein, esculin and esculetin.

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