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Simultaneous determination of iridoid glycosides and flavanoids in Lamionphlomis rotate and its herbal preparation by a simple and rapid capillary zone electrophoresis method

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Iridoid glycosides and flavanoids are two main effective components of Lamiophlomis rotata (Benth.) kudo. However, there is no method for simultaneous analysis of iridoid glycosides and flavanoids in *L. rotata* and its pharmaceutical preparations. A simple and rapid capillary zone electrophoresis (CZE) method was developed and validated for simultaneous determination of two iridoid glycosides (8-O-acetylshanzhiside methylester and 8-deoxyshanzhiside) and three flavanoids (apigenin, quercetin and luteolin) in *L. rotata*. Operational variables, such as the voltage, buffer concentration and pH were optimized, the final optimum separation condition was 10 mM sodium tetraborate-20 mM NaH_2PO_4 (pH 8.5)-15% (v/v) methanol, 238 nm UV detection, 18 kV applied voltage. The linearity and the recovery of the proposed method were very satisfactory (correlation coefficients were 0.9994–0.9998 and the recoveries were 94.5–108.8% for the analytes) and the method allowed analytes in real samples to be determined within 9 min. The proposed CZE method can be used for quality control of iridoid glycosides and flavanoids in *L. rotata* and its herbal preparation.

Keywords: CZE; *Lamiophlomis rotata* (Benth.) kudo; iridoid glycosides; flavanoids

Introduction

Lamiophlomis rotata (Benth.) kudo, which is the only species of the genus *Lamiophlomis* kudo (Labiatae), is widely spread over high mountainous regions in Qinghai and Tibet in China.[1] *L. rotata* has been used for thousands of years by Tibetans, Mongolians and Na–Xi nations to promote blood circulation, remove blood stasis, subdue swelling, alleviate pain, and reinforce marrow.[2–6] *L. rotata* has attracted increasing interest in recent years due to its prominent therapeutic effects on many diseases.[7–11] Preparations of *L. rotata* such as Duiyiwei capsule are quite frequently used to alleviate pain, subdue swelling, and stop bleeding after operations in dentistry, surgery, and gynecology.[2] Iridoid glycosides[12–19] and flavanoids[4,20] are two main effective components of *L. rotata*. The main iridoid glycosides in *L. rotata* include 8-O-Acetylshanzhiside methylester, 6-O-acetylshanzhiside methylester, shanzhiside methylester, 8-deoxyshanzhiside, phlomiol, phloyoside I and phloyoside II. Luteolin, luteolin-7-O-glicoside, quercetin, quercetin-3-Oarabinoside, apigenin and apigenin-7-O- neohesperidoside were reported as the main flavanoids in this plant. Iridoid glycosides accelerate coagulation of blood and hemostasis;[10,17] they also have antinociceptive and anti-inflammatory activities.[11] Flavanoids in *L. rotata* have the properties to promote blood circulation to remove blood stasis, detumescence, and acesodyne.[4,13]

So it's necessary to establish methods for quality control of iridoid glycosides and flavanoids in *L. rotate* and its herbal preparations. Xu *et al*.[21] developed spectrophotometry and high performance liquid chromatography (HPLC) method for determinating total flavanoids and luteolin in Duyiwei tablets, respectively. Zhang *et al*.[22] developed spectrophotometry and RP-HPLC method for determination of total flavanoids and luteolin in *L. rotate*, respectively. TLC and HPLC[23] methods were also developed to determine luteolin in *L. rotate* and its herbal preparation. Ma *et al*.[24] developed an HPLC method for determination of luteolin and isorhamnetin in Duyiwei Capsules. Luo *et al*.[25] developed an HPLC method for determination of quercetin, luteolin, and apigenin in *L. rotate* and Duyiwei capsules. A capillary electrophoresis (CE) method[26] was also developed for determination of luteolin-7-O-glucoside, isorhamnetin, apigenin, luteolin, and quercetin in *L. rotate*. Several spectrophotometry and HPLC methods have also been used for determination of iridoid glycosides in *L. rotata*. Li *et al*.[27] developed a spectrophotometry method for determination of total iridoid glycosides in herbal preparations of *L. rotate*. The amount of 8-O-Acetylshanzhiside methylester in herbal preparations of *L. rotate* was determined by an HPLC method;[28] Gao *et al*.[29] developed an HPLC method

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to determine four iridoid glycosides in preparations of *L. rotate*; Fan *et al*.[30] also developed an RP-HPLC method to determine four iridoid glycosides in Lamiophlomis rotata from different producing area. However, colourimetry methods only can determine total amounts of one kind of components; the mentioned HPLC and CE methods only determined one or several compounds of one kind of components. None of these methods has been used to determine both flavanoids and iridoid glycosides simultaneously in *L. rotate*. Therefore, there is still a need for the development of other simple, reliable, and rapid methods for the simultaneous analysis of iridoid glycosides and flavanoids in *L. rotata* and its pharmaceutical preparations.

Although CE have some limitations, such as confined sample throughput and precision because of its discontinuous sample introduction mode, it has more advantages such as high resolution, high speed of analysis, high resolution capability, small sample volume required, low costs, robustness, and reproducible analysis of highly complex samples compared with HPLC,[31,32] so it has been widely used in applications in active herbal composition separation.[33–39] Furthermore, there have been several reports in which some iridoids in other plant samples were successfully determined by CE procedures,[40–43] so CE may be a potential way for simultaneous determination of iridoid glycosides and flavanoids in *L. rotate* and its pharmaceutical preparation.

The aim of the present study was to establish a simple and rapid capillary zone electrophoresis (CZE) method for simultaneous determination of two iridoid glycosides (8-O-acetylshanzhiside methylester and 8-deoxyshanzhiside) and three flavanoids (apigenin, quercetin and luteolin) in *L. rotate* and its pharmaceutical preparation.

Experimental

Chemicals and materials

Standards of 8-O-acetylshanzhiside methylester and 8-deoxy-shanzhiside were offered by the Department of Pharmacy, Lanzhou General Hospital of PLA (58 Xiaoxihu Road, Qilihe District, Lanzhou, China). Their structures have been identified by MS and NMR spectroscopy; their purity has been identified by HPLC unitary method (purity>98%). Standards of apigenin, quercetin, and luteolin (purity>99%) were obtained from Shanghai Yousi Biotechnology Company (457 Yueluo Road, Baoshan District, Shanghai, China). The crude drug of *Lamiophlomis rotata* (Benth.) kudo was offered by the Department of Pharmacy, Lanzhou General Hospital of PLA. The herbal samples of L. rotatawere collected in alpine steppe in Maqv County (east of Qinghai–Tibet Plateau, 3400 m altitude), China. The raw material was identified by Prof. Zhigang Ma, Pharmacy College of Lanzhou University, China. Duyiwei capsule was purchased from a local drug store. All other chemicals were of analytical reagent grade. All solutions and buffers were made in distilled water. Distilled water was used in all the experiments.

Solutions preparation

Stock standard solutions of 8-O-acetylshanzhiside methyleste (650 μg/mL), 8-deoxyshanzhiside(650 μg/mL), apigenin(550 μg/mL), quercetin(450 μg/mL) and luteolin(400 μg/mL) were prepared in 80% ethanol. Working standard solutions were prepared from stock standard solutions with 80% ethanol. The running buffer contains 10 mM sodium tetraborate-20 mM NaH_2PO_4-15% methanol (pH 8.5). The running buffer was prepared daily from stock solution of 0.1 M sodium tetraborate and NaH_2PO_4. The running buffer was adjusted to the desired pH with 0.01M NaOH. All solutions were filtered through JN6 nylon syringe filters ($\Phi = 25$ mm) with 0.45 μm PTFE filter membrane before use.

Sample preparation

Ten grams of dried crude drug of Lamiophlomis rotata (Benth.) kudo were crushed by muller to obtain a fine powder and mixed well; accurately weighed 2 g powder, dissolved with 20 mL 80% ethanol and heated with circumfluence (81 °C) for 30 min. The powder of 20 capsules of Duyiwei capsule was mixed well; the weighed powder equivalent to the weight of four capsules (1.2 g) was dissolved with 20 mL 80% ethanol and heated with circumfluence (81 °C) for 30 min. The both extracted solutions were filtered through 0.45 μm syringe filters and directly injected into the CE equipment without dilution.

Apparatus

A CL1030 capillary system was used for experiment, which was produced by Beijing Cailu Science Instrument Company (Room 343, 15 Cuiweizhongli, Haidian District, Beijing, China). Uncoated silica separation capillaries of 53 cm (44.2 cm effective length) ×75 μm ID×375 μm OD (Yongnian Optical Fiber Factory, Hebei, China) were used throughout the experiment. UV detection was carried out at 238 nm. The data acquisition was carried out with an HW-2000 Chromatography Workstation (Shanghai Qianpu Software Company, Shanghai, China). Samples were introduced from the end of the capillary by hydrodynamic injection, where the sample vial was raised by 15.5 cm for 5 s. At the beginning of each working day, the capillary was flushed sequentially with 0.5 M NaOH for 10 min, distilled water for 10 min, and the running electrolyte for 10 min. A PHS-10A acidity meter (Xiaoshan Science Instrumentation Factory, Zhejiang, China) was used for the pH measurement.

Results and discussion

Optimization of CZE separation conditions

In order to ensure the highest possible sensitivity and selectivity, CZE separation conditions were optimized by studying the effects of concentration of sodium dihydrogen phosphate, methanol, pH, and applied voltage on the migration time and resolution between adjacent peaks. The peak sequence of the compounds in CZE was 8-O-acetylshanzhiside methylester, 8-deoxyshanzhiside, apigenin, quercetin, and luteolin. The identity of the recorded peaks was confirmed by comparing the migration time and adding the pure standard compounds.

According to Morin's investigation,[44] the sodium tetraborate-phosphate buffer system was chosen to get better separation of three flavanoids. In this system, different flavanoid compounds could combine with sodium tetraborate to form anion complexes with different charges and molecular weights, therefore, their migration times were different in CZE separation and could be separated completely. In order to improve the resolution of analytes, the influence of concentrations of sodium dihydrogen phosphate on the migration times and resolutions of the peaks was investigated by changing the amount of NaH_2PO_4 from 0 to 25 mM using 10 mM sodium tetraborate and 15% methanol. The

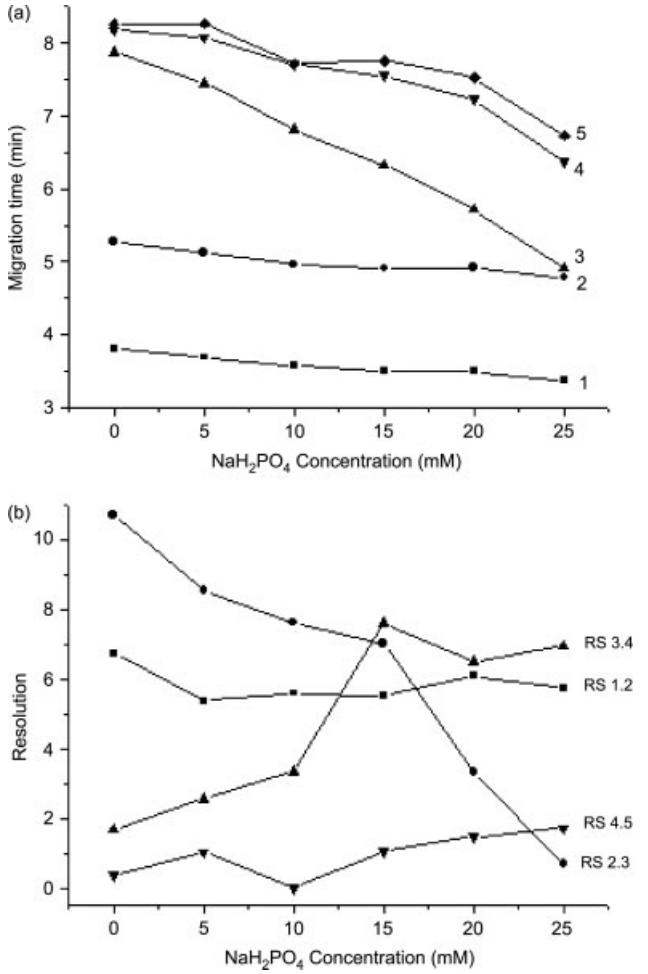

Figure 1. Influence of NaH2PO4 concentration on the migration time (a) and resolution (b) of the peaks. 1, 8-O-acetylshanzhiside methylester; 2, 8-deoxyshanzhiside; 3, apigenin; 4, quercetin; 5, luteolin. Conditions: 75 μm I.D. × 375 μm O.D. × 53 cm length (44.2 cm effective length), uncoated; Buffer: 10 mM sodium tetraborate and 15% (v/v) methanol. Voltage, 20 kV; detection wavelength, 238 nm; sample: 250 μg/mL 8-O-acetylshanzhiside methylester; 350 μg/mL 8- deoxyshanzhiside; 25 μg/mL apigenin; 50 μg/mL quercetin; 5 μg/mL luteolin.

result is shown in Figure 1. It was found in the experiments that the addition of NaH_2PO_4 changed the resolutions and migration times of iridoid glycoside compounds little; however, it could change the resolutions of three flavanoid compounds remarkably. Although 25 mM NaH_2PO_4 could get shorter migration times and higher resolution of peak 4 and 5, the resolution of peak 2 and 3 became too low. In order to get better resolution and shorter migration time, 20 mM NaH_2PO_4 was selected for the following experiments.

The effect of methanol concentrations on the separation behaviours of analytes was investigated by changing the amount of methanol from 0 to 20% using 10 mM sodium tetraborate-20 mM NaH_2PO_4. It was found in the experiments that there was little influence of methanol concentration on resolutions of standard analytes; however, the resolutions between analytes and the adjacent peaks in the herb and herbal preparation were improved effectively. At the same time, further increasing methanol concentration would make the analysis time longer. Thus 15% methanol was chosen considering the resolution and total analysis time.

To improve the resolutions, the effects of pH on the resolutions and migration time of analytes were investigated when 10 mM sodium tetraborate-20 mM NaH_2PO_4 was used as the running buffer in the pH range 7.0–9.0. The results showed that there was little influence of pH on resolutions of analytes; however, pH 8.5 could get a shorter analysis time, thus pH 8.5 was selected as the optimum condition.

The influences of separation voltages were also investigated by using different applied voltages ranging from 15 to 25 kV. As may be expected, increasing the voltage gave shorter migration times, but this also caused a decrease of resolution. With consideration for the peak shape, migration time, and resolution, the separation voltage finally chosen was 18 kV.

The final optimum separation condition was: 10 mM sodium tetraborate-20 mM NaH_2PO_4 (pH 8.5)-15% (v/v) methanol, 238 nm UV detection, 18 kV applied voltage.

Linearity, reproducibility, and limits

Under optimum conditions, the linear relationships between the peak area of the analytes and the corresponding concentrations were established by running six standard solutions for the analytes; the results are shown in Table 1.

The detection limit was estimated from the calibration curve of peak height *versus* standard concentration, and based on the concentration necessary to yield a net height equal to three times the SD of the baseline noise. The baseline noise was evaluated by recording the detector response every 5 s over a period of about 2 min. The detection limits are also given in Table 1.

The repeatability of migration times and peak areas of the five analytes in the experiment were determined by repeated injection ($n = 6$) of a standard mixture solution containing 216.7 μg/mL 8-O-acetylshanzhiside methylester, 216.7 μg/mL 8-deoxyshanzhiside, 183.3 μg/mL apigenin, 133.3 μg/mL quercetin

Table 1. The results of regression analysis and the detection limits

Analytes	Regression equation, $y = a + bx$[a]	Correlation coefficient	Linear range (μg/mL)	Detection limit[b] (μg/mL)
8-O-acetylshanzhiside methylester	$y = 10462 + 129x$	0.9998	15-650	9.2
8-deoxyshanzhiside	$y = -245 + 149x$	0.9995	5-650	3.3
apigenin	$y = 1728 + 495x$	0.9994	5-550	2.7
quercetin	$y = -984 + 661x$	0.9994	5-400	3.8
luteolin	$y = 3083 + 918x$	0.9994	5-450	2.6

[a] y and x are the peak areas and the concentration of the analytes, respectively.
[b] The detection limit was defined as the concentration where the signal-to-noise ratio is 3.

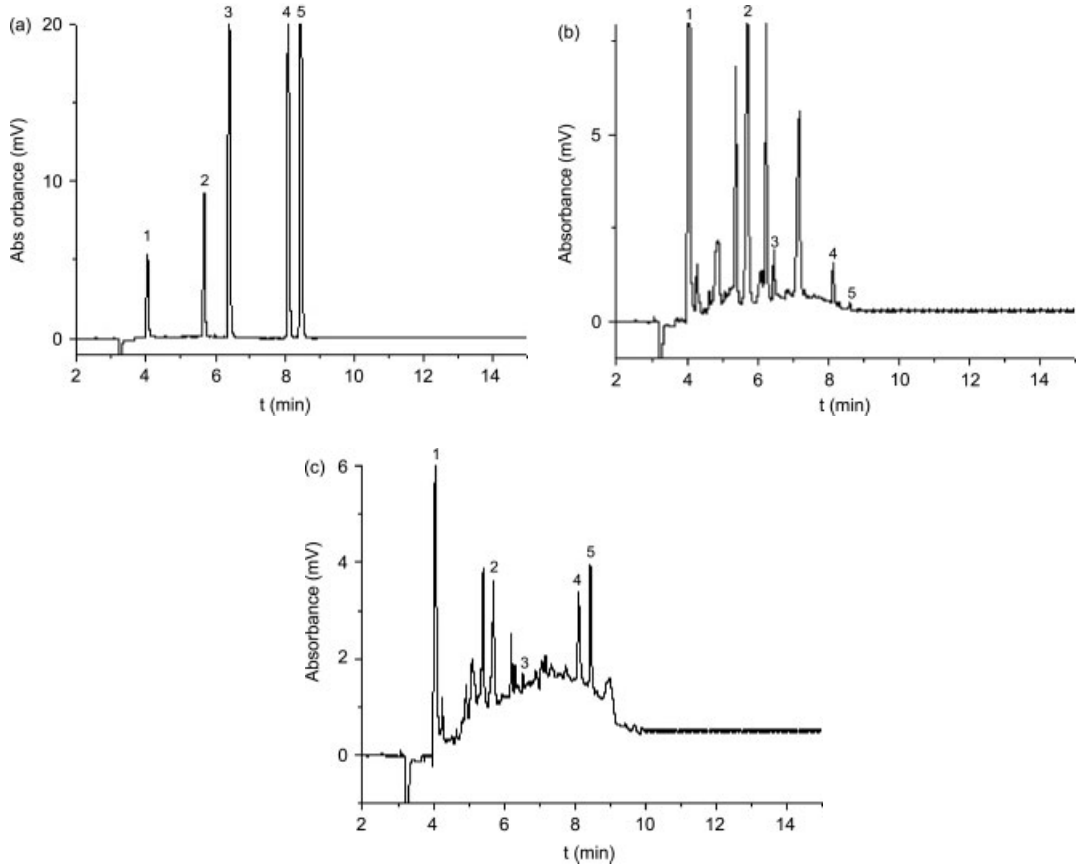

Figure 2. Electrochromatograms of a standard mixture sample: 216.7 μg/mL 8-O-acetylshanzhiside methylester, 216.7 μg/mL 8-deoxyshanzhiside, 183.3 μg/mL apigenin, 133.3 μg/mL quercetin and 150 μg/mL luteolin (a), Lamionphlomis rotate (b) and Duyiwei capsule (c). 1, 8-O-acetylshanzhiside methylester; 2, 8-deoxyshanzhiside; 3, apigenin; 4, quercetin; 5, luteolin. Buffer: 10 mM sodium tetraborate-20 mM NaH2PO4 (pH 8.5)-15% (v/v) methanol. Voltage, 18 kV. Other conditions are the same as in Figure 1.

Table 2. Contents of the five components from *Lamionphlomis rotate* and its herbal preparation by calibration and standard addition methods (n=3)

	Lamionphlomis rotate				Duyiwei capsule			
Samples	Calibration	RSD (%)	Standard addition	RSD (%)	Calibration	RSD (%)	Standard addition	RSD (%)
8-O-acetylshanzhiside methylester	40.1 mg/g	2.6	40.4 mg/g	2.0	8.72 mg/capsule	2.1	8.75 mg/capsule	2.2
8-deoxyshanzhiside	18.9 mg/g	2.3	19.1 mg/g	2.1	2.36 mg/capsule	1.9	2.30 mg/capsule	2.3
apigenin	0.35 mg/g	2.8	0.33 mg/g	2.9	5.56 μg/capsule	3.0	5.58 μg/capsule	3.2
quercetin	0.46 mg/g	3.3	0.48 mg/g	3.4	409 μg/capsule	3.9	413 μg/capsule	3.9
luteolin	7.5 μg/g	4.1	8.0 μg/g	4.0	183 μg/capsule	4.2	188 μg/capsule	4.0

and 150 μg/mL luteolin. The electropherogram of a standard mixture is shown in Figure 2A. The relative standard deviation (RSD) values of the migration times and peak areas were 1.9% and 2.1% for 8-O-acetylshanzhiside methylester, 1.7% and 2.4% for 8-deoxyshanzhiside, 2.1% and 3.5% for apigenin, 1.6% and 2.2% for quercetin, 2.6% and 3.2% for luteolin (intra-day), 2.3% and 3.1% for 8-O-acetylshanzhiside methylester, 2.4% and 3.4% for 8-deoxyshanzhiside, 2.7% and 3.8% for apigenin, 2.6% and 3.2% for quercetin, 3.7% and 4.2% for luteolin (inter-day), respectively.

Analysis of herbal samples

To test the applicability of the developed method based on CZE, quantitative analysis was performed under the optimum conditions obtained from the experiments described above. The method was applied to the analysis of 8-O-acetylshanzhiside methylester, 8-deoxyshanzhiside, apigenin, quercetin and luteolin in *Lamionphlomis rotate* and Duyiwei capsule. The contents of the analytes are given in Table 2. The typical electropherograms of *Lamionphlomis rotate* and Duyiwei capsule are shown in Figures 2B and 2C. The peaks were identified by comparing the migration time with the standards and adding the pure standard compounds to the real sample solutions. At the same time, the standard addition technique was applied to the same preparations which were analyzed by calibration curve of methods. There was no difference between calibration curve of methods and standard addition techniques. These results showed that there was no interference

Table 3. Recovery of the five analytes (n=3)

Sample	component	Added (μg/mL)	Found (μg/mL)	Recovery (%)	RSD (%)
Lamionphlomis rotate	8-O-acetylshanzhiside methylester	325	317.8	97.8	3.1
	8-deoxyshanzhiside	325	327.1	100.6	2.8
	apigenin	11	11.5	104.3	4.5
	quercetin	8	8.4	105.0	4.4
	luteolin	9	8.6	95.6	4.9
Duyiwei capsule	8-O-acetylshanzhiside methylester	130	135.5	104.2	4.7
	8-deoxyshanzhiside	65	59.8	99.7	3.2
	apigenin	11	10.4	94.5	4.9
	quercetin	8	7.6	95.0	4.3
	luteolin	9	9.8	108.8	2.4

Table 4. A comparison of deferent methods to determinate iridoid glycosides and flavanoids in *L. rotate*

Reference	method	Analytes	Mobile phase/buffer/ color deveploping agent	Analysis time (min)	λ (nm)
–	Presented method(CZE)	8-O-acetylshanzhiside methylester; 8-deoxyshanzhiside; apigenin; quercetin; luteolin	10 mM sodium tetraborate-20 mM NaH_2PO_4 (pH 8.5)-15% (v/v) methanol	9	238
[21]	HPLC;	luteolin;	methanol-0.5% H_3PO_4(50 : 50)5% sodium nitrite-10%	12	350;
	Spectrophotometry	total flavonoids	aluminum nitrate	–	500
[22]	RP-HPLC;	luteolin;	methanol-0.5% H_3PO_4(55 : 45) 5% sodium nitrite-10%	17	350;
	Spectrophotometry	total flavonoids	aluminum nitrate		500
[23]	HPLC;	luteolin	benzene-ethylacetate-acetic acid (6 : 4:0.5)	24	365
	TLC	luteolin	benzene-ethylacetate-acetic acid (6 : 4:0.5)	–	350
[24]	HPLC	luteolin isorhamnetin	methano1 : water(50 : 50), pH3.0	20	360
[25]	HPLC	quercetin; luteolin; apigenin	methanol- water (1 mmol·L hexadecyl-trimethylammonium bromide)(61 : 39), pH3.5	16	370
[26]	CE	luteolin-7-O-glucoside, isorhamnetin, apigenin, luteolin and quercetin	30 mM borate-8% (v/v) acetonitrile pH9.0	25	210
[27]	spectrophotometry	total iridoid glycosides	paradimelhylaminobenzal dehyde	–	620
[28]	HPLC	8-O-acetylshanzhiside methyl ester	(acetonitrile-water)	10	234
[29]	HPLC	sesamoside; shanzhiside methylester; 7,8 - dehydr openstemoside;8-O-acetylshanzhiside methyl ester	gradient elution (methanol-water)	22	350
[30]	RP-HPLC	shanzhiside methylester; phloyosid II; loganin methylester; 8-O-acetylshanzhiside methyl ester	gradient elution (acetonitrile-water)	35	235

from the matrix. The results are presented in Table 2. The recovery of the method was determined with the addition of the standards in the real sample solution; the results are given in Table 3. These show that the method had high efficiency and stability.

The comparison of the present method with others is shown in Table 4. It can be seen that the present method had higher efficiency and higher speed of analysis especially compared with HPLC and could get very satisfactory results.

Conclusions

In this paper, a simple CZE method has been successfully used for simultaneous determination of two iridoid glycosides (8-O-acetylshanzhiside methylester and 8-deoxyshanzhiside) and three flavanoids(apigenin, quercetin and luteolin) in traditional Tibetan herb *Lamionphlomis rotate* and its herbal preparation for the first time. The separation could be achieved within 9 min

under optimum conditions described above. The resolution of the analytes and the recovery of the method were both satisfactory. The proposed CZE method could be a good alternative for quality control of *L. rotate* and its herbal preparations.

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