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Short communication

Simultaneous determination of four bioactive constituents in Liuwei Dihuang Pills by micellar electrokinetic chromatography

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Abstract

A micellar electrokinetic chromatography (MEKC) method for the simultaneous determination of four bioactive constituents (morroniside, loganin, paeoniflorin and paeonal) in the Chinese patent medicine Liuwei Dihuang Pills is established. A carrier composed of 0.2 M boric acid, 0.02 M sodium dodecyl sulfate (SDS) and 5% acetonitrile (pH was adjusted to 10.5 with 0.1 M NaOH) is found to be the most suitable electrolyte for this separation. The four constituents in Liuwei Dihuang Pills can be easily determined within 16 min. Optimization of separation is realized with the univariate approach by studying the effects of four factors relevant to run buffer on migration times.

Keywords: Liuwei Dihuang Pills; Micellar electrokinetic chromatography; Morroniside; Loganin; Paeoniflorin; Paeonal

1. Introduction

Liuwei Dihuang Pills, one of the most important Chinese patent medicines, are widely used in eastern Asia. In China, there are hundreds of medicinal manufacturers who produce Liuwei Dihuang Pills and its derivative varieties, such as Zhibai Dihuang Pills, Guifu Dihuang Pills, Mingmu Dihuang Pills, Qiju Dihuang Pills, Maiwei Dihuang Pills, Guishao Dihuang Pills, etc. Suitable assay methods are therefore needed urgently for quality control purpose. Several methods have been developed to determine one constituent in Liuwei Dihuang Pills. Determination of paeonal by gas chromatography (GC) [1] and high performance liquid chromatography (HPLC) [2,3], ursolic acid by thin layer chromatography (TLC) [4] and HPLC [5] are commonly used. A HPLC method combined with principal component analysis (PCA) was reported by Zhang et al.[6]. However, this HPLC method using gradient elution needs at least 90 min for a single run. Efforts to develop a simpler and facile analytical method that can assay as many bioactive constituents as possible are quite necessary. Capillary electrophoresis (CE) in its modern form was first described by Joegenson and Luckacs in 1981 [7,8], its application to the separation and determination of a variety of samples has become increasingly widespread because of its minimal sample volume requirement, short analysis time and high separation efficiency. The simultaneous determination of paeonal and paeoniflorin in Liuwei Dihuang Pills by micellar electrokinetic chromatography (MEKC) was reported by Liuand Gao [9]. The optimum separation conditions were found to be 0.02 M borax, 0.03 M SDS and 20% acetonitrile at a pH level of 9.30. The UV detection was set at 203 nm. However, the UV detection at 203 nm gave a poor baseline and an interferential separation. In this study, we established a MEKC method for the simultaneous determination of four bioactive constituents in Liuwei Dihuang Pills. The method could be more comprehensive in the assessing of the Liuwei Dihuang Pills inner quality compared with the determination of one or two constituents. Furthermore, there is no report of the simultaneous determination of three or four constituents in Liuwei Dihuang Pills by any kind of analytical method to date.

Liuwei Dihuang Pills are composed of *Radix Rehmannide Preparata*, *Rhizoma Dioscoreae*, *Fructus Corni, Cortex Moutan, Rhizoma Alismatis* and *Poria*. They have the therapeutic function of protecting the liver, stopping bleeding, inducing diuresis, blocking inflammation and fungi, lowering blood sugar level and reinforcing the function of the heart [10]. Morroniside (1),

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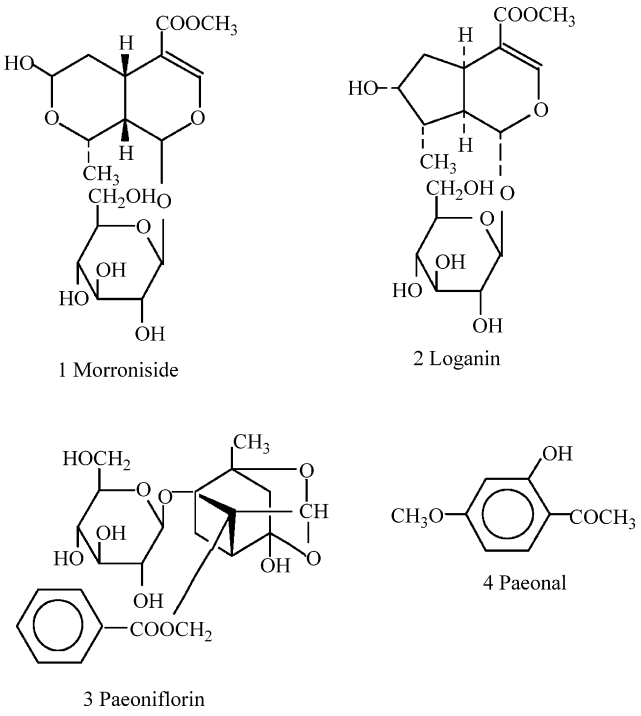

Fig. 1. Structures of the four marker substances.

loganin (2), paeoniflorin (3) and paeonal (4) are four bioactive constituents of Liuwei Dihuang Pills, their structural formulae are shown in Fig. 1. Morroniside and loganin are iridoid glycosides, they have been shown to possess the therapeutic effects of nourishing, astringing and inhibiting bacteria. Paeoniflorin and paeonal have the effects of inhibiting bacteria and lowering blood pressure. Using these constituents as marker substances, a CE method for appraising the quality of Liuwei Dihuang Pills was developed. This simple method is sensitive, reliable and efficient. It is not only a way of evaluating the quality of Liuwei Dihuang Pills, but also an excellent alternative method in quality control for medicinal manufacturers.

2. Experimental

2.1. Reagents and materials

Morroniside and loganin were purchased from the Zhongriyouhao Hospital (Beijing, China). Paeoniflorin, paeonal and chloramphenicol (used as internal standard) were provided by the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Boric acid and sodium dodecyl sulfate (SDS) were from Shenyang Chemical Reagent Factory (Shenyang, China). Acetonitrile was of the HPLC grade (Yuwang Chemical Reagent Factory, Shandong, China). All the aqueous solutions were made up with doubly distilled water. Other chemicals were of the analytical grade.

2.2. Apparatus and conditions

The experiments were performed on a Unimicro electrophoresis instrument (Unimicro, Tianjin, China). A fused silica capillary (75 μm i.d., 375 μm o.d.), length 60 cm, effective length 50 cm to detector (Yongnian Photoconductive Fibre Factory, Hebei, China) was used. The conditions were as follows: applied voltage, 14 kV; run time, 20 min; UV detection at 240 nm; temperature, 20 °C. A pHs-25 pH meter (Shanghai Analytical Instrumentation Factory, Shanghai, China) was used for pH measurements. The electrolyte was a buffer solution consisting of 0.2 M boric acid, 0.02 M SDS and 5% acetonitrile (v/v), which was adjusted to 10.5 with 0.1 M NaOH. The following rinses were used after each sample: water 1 min, 0.1 M NaOH 1 min, water 1 min, running buffer 1 min. Each morning, the capillary was regenerated by rinsing it with 0.1 M NaOH 10 min, water 10 min and buffer solution 10 min. This was also done when the capillary was changed.

2.3. Sample preparation

Five grams of powder of Liuwei Dihuang Pills were accurately weighed and extracted with 50 ml of 95% ethanol for 0.5 h in an ultrasonic bath. The extraction was repeated three times. The extracted solutions were combined and concentrated nearly to dryness. After the addition of a 5 ml of internal standard solution (10 mg of chloramphenicol in 25 ml of 95% ethanol), the extract was diluted to 100 ml with 95% ethanol and filtered through 0.45 μm filter before sample injection.

3. Results and discussion

3.1. Analytical conditions

Before undertaking the MEKC experiments, preliminary studies using the capillary zone electrophoresis (CZE) were attempted. Different compositions of the electrolyte solution were tested for optimization. The peaks of morroniside and loganin were always crowded together with serious overlapping. MEKC was first reported by Terabe et al. in 1984 [11], and since then, it has been applied successfully to both charged and neutral compounds [12–16]. A negatively charged surfactant such as sodium dodecyl sulfate (SDS) or sodium decyl sulfate was usually added to the background electrolyte to improve the selectivity of separation [17]. A few reports have been published on capillary electrophoretic analysis of iridoid glycosides showing that several iridoids can be successfully separated by MEKC [18,19]. The final composition of the electrolyte solution was 0.2 M boric acid, 0.02 M SDS, 5% acetonitrile, 0.1 M NaOH adjusted to pH 10.5. In this case, the four constituents could easily be separated.

3.2. Effect of boric acid concentration

In the presence of 0.02 M SDS, 5% acetonitrile (v/v) and pH 10.5, a range of 0.05–0.3 M boric acid were used in order to study the effect of boric acid concentration on the separability. The migration times and the overall resolution of all the constituents increase with increasing buffer concentrations. Higher buffer concentration also led to a higher viscosity coefficient of the solution.A quantity of 0.2 M boric acid was found to produce

a good resolution. Too high a buffer concentration decreased the resolution, probably because of the increase of the Joule heat.

3.3. Effect of SDS

In the presence of 0.2 M boric acid, 5% acetonitrile (v/v) and pH 10.5, a range of 0.01–0.05 M SDS were used in order to study the effect of SDS concentration on the separability. The migration times of all the constituents increase with increasing SDS concentrations. The four constituents were completely separated from 0.02 to 0.05 M SDS, however, higher SDS concentration gave a poor baseline and prolonged the analysis time. Therefore, 0.02 M SDS was chosen in this work.

3.4. Effect of acetonitrile

Different organic solvents were tested for optimization. Acetonitrile was selected as modifier because of the sharper peaks, smoother baseline and alternation of selectivity and improvement of resolution. Typically, less than 10% (v/v) of organic modifiers were used to avoid breaking down the micellar structure [20]. In the presence of 0.2 M boric acid, 0.02 M SDS and pH 10.5, a range of 0–10% (v/v) acetonitrile were used to study the effect of acetonitrile concentration on the separability. The results obtained are given in Fig. 2. The migration times of compounds 1, 2, and 3 increased with the concentration of acetonitrile within the experimental range, due to the decrease of electro-osmotic flow caused by the addition of acetonitrile. On the contrary, the migration time of compound 4 (paeonal) became shorter probably because the solubilization of paeonal by the micelle decreased. With an increase of the concentration of acetonitrile, the adsorption and diffusion of analytes were decreased because the dissociation of silanol in the inner wall of the capillary and the current were decreased. These led to the increase of plate number [21]. Thus, addition of acetonitrile to the buffer solution made the peaks sharper, the baseline smoother and also produced a better separation.

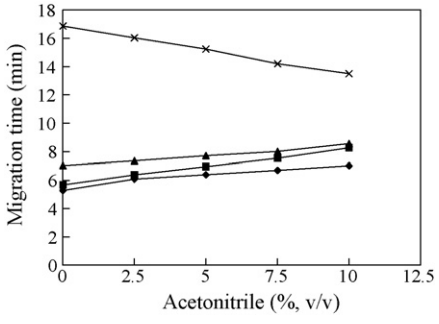

Fig. 2. The effects of acetonitrile concentration on migration times. ♦ Morroniside; ■ loganin; ▲ paeoniflorin; × paeonal. Electrophoresis conditions: 0.2 M boric acid, 0.02 M SDS, pH 10.5, with various volume percentage of acetonitrile, fused silica capillary (75 μm i.d., 375 μm o.d.), length 60 cm, effective length 50 cm to detector; applied voltage, 14 kV; run time, 20 min, UV detection at 240 nm; temperature, 20 °C.

3.5. Effect of pH

pH is the most powerful method for separation in CE as well as in HPLC. The pH of the buffer solution affects electro-osmotic flow as well as the overall charge of the analyte. The effects of pH on migration times were investigated from pH 8.5 to 11 in the presence of 0.2 M boric acid, 0.02 M SDS and 5% acetonitrile (v/v). The results obtained are shown in Fig. 3. The migration times decreased with the increasing of the buffer pH for all the constituents. This is probably due to two reasons: first, the electro-osmotic flow increased with buffer pH, which led to a decrease of the migration times of the analytes; second, the complex formation reaction is a strongly pH-dependent equilibrium. The complex formation becomes greater at higher pH values and the dissociated forms escaped being retained by the micelle. At pH 10.5, the four constituents can be well separated within a relatively short time.

From the above results, a buffer solution containing 0.2 M boric acid, 0.02 M sodium dodecyl sulfate, 5% acetonitrile and pH 10.5 was chosen. The electropherogram of Liuwei Dihuang Pills is shown in Fig. 4. The four constituents could be successfully determined within 16 min.

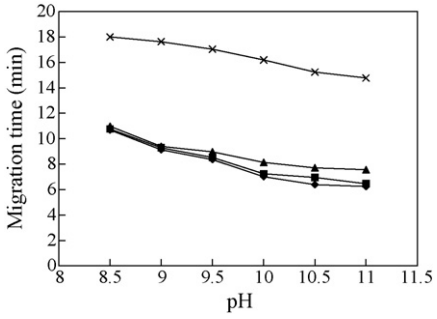

Fig. 3. The effects of pH on migration times. ♦ Morroniside; ■ loganin; ▲ paeoniflorin; × paeonal. Electrophoresis conditions: 0.2 M boric acid, 0.02 M SDS, 5% acetonitrile (v/v), with various pHs and other conditions as in Fig. 2.

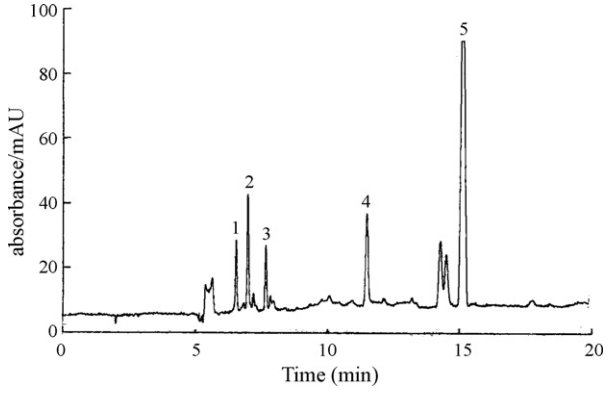

Fig. 4. Electropherogram of Liuwei Dihuang Pills. (1), Morroniside; (2), loganin; (3), paeoniflorin; (4), chloramphenicol (internal standard) and (5), paeonal. Electrophoresis conditions: 0.2 M boric acid; 0.02 M SDS; 5% acetonitrile (v/v), pH 10.5 and other conditions as in Fig. 2.

Table 1
Contents of marker substances in Liuwei Dihuang Pills ($n = 5$)

Sample	Constituents	Content (mg/g)	R.S.D (%)
Beijing	Morroniside	3.3	1.71
	Loganin	2.4	1.47
	Paeoniflorin	1.5	1.63
	Paeonal	5.0	2.39
Henan	Morroniside	2.8	1.68
	Loganin	2.9	1.65
	Paeoniflorin	2.1	2.04
	Paeonal	5.1	1.99
Haerbin	Morroniside	1.9	1.47
	Loganin	2.3	1.84
	Paeoniflorin	0.9	2.25
	Paeonal	3.8	2.77
Guangzhou	Morroniside	2.3	2.48
	Loganin	2.1	1.97
	Paeoniflorin	0.7	2.02
	Paeonal	4.3	2.61

3.6. Method validation and determination of the constituents in Liuwei Dihuang Pills

The measurements of intra- and inter-day variability were utilized to assess the repeatability and reproducibility of the developed assay. The relative standard deviation (R.S.D) values of the peak area ratios for five replicate injections intra-day were 1.21% for morroniside, 0.92% for loganin, 1.35% for paeoniflorin, and 1.72% for paeonal, respectively. The inter-day variability was examined over three days by performing five replicates each day. The R.S.D values inter-day were 1.47% for morroniside, 1.68% for loganin, 1.53% for paeoniflorin, and 2.47% for paeonal. The extraction recovery was tested by adding known amounts of the four constituents. The mean recoveries ($n = 5$) of morroniside, loganin, paeoniflorin and paeonal were 101.2, 101.8, 98.4 and 102.5%, respectively, and the R.S.D values were 2.7, 3.1, 2.3 and 3.6%, respectively. All the tailing factors of the peaks were very close to 1. The plate numbers of the peaks were 8.9×10^4 for morroniside, 1.0×10^5 for loganin, 1.3×10^5 for paeoniflorin, and 2.2×10^5 for paeonal. The limits of detection (LOD) and quantification (LOQ) were separately determined in five replicate determinations at a signal-to-noise ratio (S/N) of 3 and 10, respectively. The LOD were 2.2 μg/ml for morroniside, 1.7 μg/ml for loganin, 0.6 μg/ml for paeoniflorin and 0.3 μg/ml for paeonal. The LOQ were 7.3 μg/ml for morroniside, 5.7 μg/ml for loganin, 2.0 μg/ml for paeoniflorin and 1.0 μg/ml for paeonal. Calibration graph (peak-area ratio, y, versus concentration, x, μg/ml) was constructed in the range 15.6–312.0 μg/ml for morroniside, 18.2–364.0 μg/ml for loganin, 7.0–175 μg/ml for paeoniflorin and 44.2–663.0 μg/ml for paeonal. The regression equations of these curves and their correlation coefficients were calculated as follows: morroniside, $y = 0.051 \times -0.487$ ($r = 0.9994$); loganin, $y = 0.0295 \times -0.2339$ ($r = 0.9997$); paeoniflorin, $y = 0.0972 \times -0.0267$ ($r = 0.9998$); paeonal, $y = 0.0566 \times -2.197$ ($r = 0.9998$). Liuwei Dihuang Pills from four medicinal manufacturers (Beijing, Henan, Haerbin and Guangzhou) were determined. The contents of morroniside, loganin, paeoniflorin and paeonal are shown in Table 1.

4. Conclusion

A MEKC method for simultaneous determination of four bioactive constituents in Liuwei Dihuang Pills was established. The method is simple and fast with excellent resolution and reproducibility, which provided a good way for evaluating the quality of Liuwei Dihuang Pills.

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