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Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 365–370
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Determination of icariin and metabolites in rat serum by capillary zone electrophoresis: rat pharmacokinetic studies after administration of icariin☆

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Received 23 October 2003; received in revised form 28 June 2004; accepted 30 June 2004
Available online 17 August 2004

Abstract

A simple and rapid method for determination of icariin (ICA) and its two metabolites, icaritin (ICT) and desmethylicaritin (DICT), by capillary zone electrophoresis has been developed and validated. Optimum separation of ICA, ICT, and DICT was obtained on 43.6 cm × 50 μm capillary using sodium tetraborate (30 mmol/L), monobasic sodium phosphate (50 mmol/L)–acetonitrile (50:50, v/v) (pH 10.0) as running buffer. Carbamazepine (CMP) was used as internal standard (IS). The temperature and voltage were optimized at 25 °C and 12 kV, respectively. The limit of detection of ICA was 1.0 mg/L (S/N = 3) by UV detection at 270 nm. The elaborated method was tested in vivo after administration of a single dose of 120 mg ICA/kg to healthy rats. Calculated parameters confirmed usefulness of the method in rat pharmacokinetic studies on ICA.
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Keywords: Icariin; Capillary zone electrophoresis; Serum deproteinization; Pharmacokinetics

1. Introduction

Herba Epimedii from many species of genus *Epimedium* (family Berberidaceae) has been widely used as a tonic, aphrodisiac, and antirheumatic in China [1]. Its effective ingredient—icariin (ICA, structure shown in Fig. 1), a typical flavonol glycoside, which gave various pharmacological effects such as immunoregulation, modulation of endocrine, amelioration of cardio- and cerebro-vascular system [2]. It has been reported that formulated medicines of Herba Epimedii could give rise to some pharmacological effects [3,4] similar to the estrogenic activities of daidzein [5,6]. The therapeutical effects of Herba Epimedii are probably due to the indicative constituent—ICA.

It was furthermore reported that flavones and isoflavones either cause or strikingly reinforce estrogen-like activities only after hydrolysis of their corresponding glycosides [7]. Other studies showed that the main structural feature of flavonoids required for estrogenicity is the presence of a mono hydroxyl group in the 4′-position of the B-ring of the flavan nucleus [8]. Two ICA-derived metabolites, icaritin (ICT) and desmethylicaritin (DICT) (structure shown in Fig. 1), have been detected in urine of rats following oral administration of ICA. Additionally, ICT and DICT significantly increased cell proliferation compared with untreated control but the parent compound ICA failed to possess this effect in MCF-7 cell proliferation assay (E-screen test) [9]. Up to now, however, little is known about the metabolites of ICA in serum. Therefore, method development for determining ICA, ICT, and DICT in serum is essential for exploration

☆ Projects supported by the National Natural Science Foundation of China, No. 30171121, Sino-German Research project of NSFC-DFG, No. GZ051-3 (147) and Key Project of Zhejiang Provincial Natural Science Foundation of China, No. 2003C24005.
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doi:10.1016/j.jpba.2004.06.021

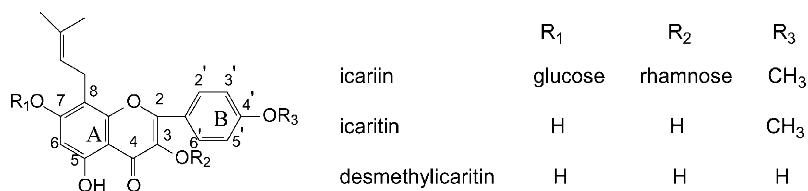

Fig. 1. The chemical structure of three studied flavonoids.

of material base of the pharmacological effects of ICA in vivo.

Generally, high-performance liquid chromatography (HPLC) was employed to analyze crude herbs or to determine several marker components in traditional Chinese medicinal preparations [10,11]. And ICA has been characterized in biological samples using HPLC [12]. But HPLC method not only is expensive, but also large amounts of toxic organic solvent, such as methanol and acetonitrile. The capillary electrophoresis (CE) has been reviewed many times, and clearly it continues to be a very active research area in separation science since this technique often provides higher resolving power, shorter analysis time and lower operating cost than HPLC. It has also been reported in the analysis of traditional Chinese medicine [13,14]. CE was, however, not applied to the separation of flavonoids until 1991 [15]. And a micellar electrokinetic capillary chromatography (MEKC) has been used to separate flavonoids (including ICA and DICT) and phenylethanoid glucoside isolated from the aerial parts of *Epimedium* species [16]. Since capillary zone electrophoresis (CZE) method is a simpler mode of CE comparing with MEKC, it has been established for determination of ICA in Herba epimedii and its preparation [17]. The purpose of the present study, therefore, was development and validation of a CZE method suitable for the determination of ICA and its metabolites in rat serum, as an alternative to HPLC, designed for pharmacokinetic study.

2. Material and methods

2.1. Materials

ICA was purchased from National Institute for the Control of Pharmaceuticals and Biological Products, Beijing, China. Carbamazepine (CMP, IS) was provided by Zhejiang Hisun Pharmaceutical Co. Ltd. Acetonitrile was HPLC grade (Siyou Co., Tianjin, China). Sodium tetraborate and monobasic sodium phosphate were analytical grade.

ICT and DICT were prepared and purified from ICA by hydrolysis and methylation, and purity was above 98% detected by HPLC.

2.2. Apparatus

The CZE analyses were performed on a BioFocus® 3000 automated capillary electrophoresis system (Bio-Rad, Hercules, CA) equipped with UV detection at 270 nm. BioFocus® 3000 software was used for instrumental control, data acquisition, and data analysis. An untreated fused-silica capillary tube (effective length, 43.6 cm × 50 μm i.d.) (Hebei Yongnian Photoconductive Fibre Factory, Hebei, China) was used for separation. Sodium tetraborate (30 mmol/L), monobasic sodium phosphate (50 mmol/L)–acetonitrile (50:50, v/v) (pH 10.0) was chosen as running buffer. The electrophoresis buffer was passed through a 0.45 μm filter and then degassed ultrasonically for 5 min before actual use. The typical analytical conditions were: voltage, 12.0 kV; injection, 10 psi s; capillary and carousel temperature were 25 and 10 °C, respectively. Analysis was conducted in the following manner: the capillary tube was rinsed first with sodium hydroxide (0.1 M) for 2 min and then with distilled water for 2 min, and was conditioned with the electrophoresis buffer for 3 min between each injection.

2.3. Preparation of standard solution

Standard stock solution of ICA (1 mg/mL) and IS (1 mg/mL) were prepared with methanol and stored at 4 °C. Working standards from the concentrated stock solutions were prepared, IS added (used as final concentration of 40 mg/L) and then diluted with 70% aqueous methanol or rat serum to yield concentrations of 2.5, 10, 50, 100, and 150 mg/L.

2.4. Sample preparation

Serum sample (0.1 mL) and 0.2 mL of acetonitrile containing the IS were mixed up vigorously for 60 s and centrifuged at 3743 × *g* for 15 min with a centrifuger (Heraeus Labofuge 400R, Germany). Then, its supernatant was poured into another sample tube. The supernatant was evaporated to dryness by blowing air at normal temperature and re-dissolved in 70% aqueous methanol of the same amount as the serum used for this preparation.

2.5. Validation of the assay

2.5.1. Linearity

A calibration curve was generated from the resulting electropherograms based on the ratio of the peak areas of ICA to IS. A standard curve for five replicates at each data point (2.5, 10, 50, 100, and 150 mg/l) was constructed and goodness-of-fit was determined by linear regression.

2.5.2. Reproducibility

Intra- and inter-day reproducibility studies were performed on five replicates, four different concentrations of ICA (in the linear range) were measured five times in a consecutive manner to obtain intra-day reproducibilities. The inter-day analyses of two different concentrations were repeated on five separate days.

2.5.3. Recovery

First series consisted of blank serum spiked with ICA to yield five different concentrations and IS (40 mg/L). The samples were extracted according to the procedure as described in Section 2.4. Then, serum samples were supplemented with IS only (second series). ICA was added to a dry residue obtained after the extraction. The dissolved samples were injected into the capillary. The recoveries were calculated as the area ratio of ICA to IS using the formula:

$$\text{recovery}\,(\%) = \frac{\text{peak}_{\text{ICA extr.}}/\text{IS}}{\text{peak}_{\text{ICA non-extr.}}/\text{IS}} \times 100$$

2.6. In vivo application—pharmacokinetic studies

The practical potential of the elaborated method of ICA determination in pharmacokinetic studies was demonstrated in healthy rats. Sprague–Dawley (SD) rats (200–220 g) were provided by the Experimental Animals Center of Zhejiang University (Hangzhou, Zhejiang, China). Animals were fasted for 18 h before experiment with free access to water. The study was approved by the local animal ethics committee. A single dose of 120 mg ICA/kg was administered by gavage to four rats (two males and two females). Serum was prepared from blood collected via caudal vein at 5, 10, 20, 30, 40, 60, 80, 120, 180, and 240 min after administration. Serum samples were prepared for analysis as described in Section 2.4. The pharmaceutical kinetics software (PKS) 1.0.2 software package (Shanghai Hongneng Software Co. Ltd., China) was used for calculation of pharmacokinetic parameters. Area under the curve ($AUC_{0\to\infty}$) was estimated by trapezoidal rule with extrapolation to infinity using the ratio C_n/K_e where C_n was the last measurable concentration. The elimination rate constant (K_e) was estimated from the terminal linear segment of the log serum concentration/time data. The elimination half life ($T_{1/2}$) was calculated from $\ln 2/K_e$. T_{max} was estimated from concentration/time curve and C_{max} was read at T_{max}. Clearance (Cl/F) of the serum drug was calculated dividing the dose (D) of icariin by $AUC_{0\to\infty}$: $\text{Cl}/F = D/AUC_{0\to\infty}$ ($\text{L kg}^{-1}\,\text{min}^{-1}$). The volume of distribution (V_d/F) was calculated using the formula: $V_d/F = D/AUC_{0\to\infty}K_e$ (L kg^{-1}).

3. Results and discussion

3.1. Optimization of separation

Due to the possible metabolites, ICT and DICT, may exist in ICA-treated rat serum, one of the major concerns of the procedure is to verify whether this method is suitable to

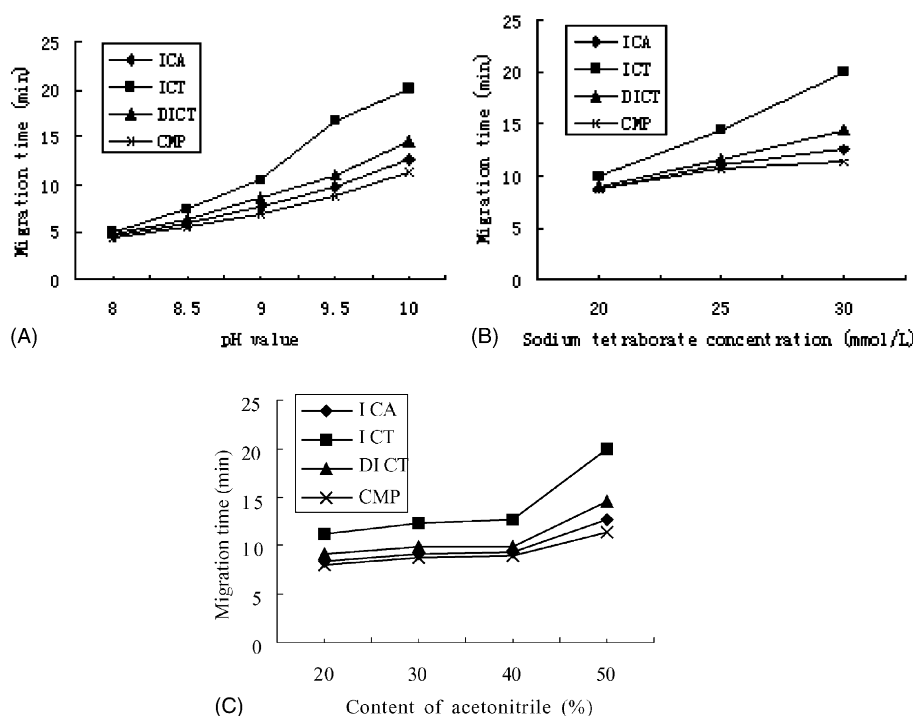

Fig. 2. Migration time of the flavonoids as a function of buffer pH (A), concentration (B), and content of acetonitrile (C). Conditions: (A) sodium tetraborate, 30 mmo/L; monobasic sodium phosphate, 50 mmol/L; 50% acetonitrile; applied voltage, 12 kV; temperature, 25 °C; UV detection wavelength, 270 nm. (B) pH 10.0; monobasic sodium phosphate, 50 mmol/L; 50% acetonitrile; applied voltage, 12 kV; temperature, 25 °C; UV detection wavelength, 270 nm. (C) pH 10.0; sodium tetraborate, 30 mmol/L; monobasic sodium phosphate, 50 mmol/L; applied voltage, 12 kV; temperature, 25 °C; UV detection wavelength, 270 nm.

determine these substances present in the serum samples. The optimization of electrophoretic conditions were done using ICA, ICT, and DICT. Separation was achieved by optimizing the pH of the buffer, sodium tetraborate, and organic modifier conentration.

3.1.1. Effect of electrolyte pH

To verify the effect of buffer pH on migration behavier, experiments were performed with 30 mmol/L sodium tetraborate, 50 mmol/L monobasic sodium phosphate, and 50% acetonitrile as background electrolyte (Fig. 2A). The result showed that the migration times of flavonoids and IS increased with the increase of pH from 8.0 to10.0 due to greater ionization of the phenolic hydroxyl groups at higher pH resulting in greater mobilities of the flavonoids in the opposite direction to electroosmotic flow (EOF), although rapid increase in EOF with the pH increase. At pH < 8.5, ICA, ICT, DICT, and IS could not separate because all flavonoids migrated together with EOF. Flavonoids as phenol types are weak acids with pK_a value of 9.0–10.0, therefore, these compounds will be constantly ionized in higher pH, and migration times and resolution will increase with increasing of pH values. The four compounds can be completely separated with moderate analysis time at pH 10.0, that was then selected as the preference pH for further optimization.

3.1.2. Effect of sodium tetraborate concentration

The effect of sodium tetraborate solution concentration ranging from 20 to 30 mmol/L on migration time under pH 10.0, 50 mmol/L monobasic sodium phosphate, 50% acetonitrile indicated that the migration times and resolution of flavonoids increased (Fig. 2B) because the increase in ionic strength reduced the EOF. Take account of the resolution, peak shape, analytical time and repeatability, 30 mM sodium tetraborate was selected as optimum concentration.

3.1.3. Effect of organic modifier conentration

It has been reported that the addition of an organic modifier to the buffer is an important parameter to improve the separation selectivity, efficiency, and resolution [18], as it results in the modification of partition coefficient, mobile phase polarity and EOF. In order to improve the resolution, addition of acetonitrile into the background electrolyte consisting of 30 mmol/L sodium tetraborate, 50 mmol/L monobasic sodium phosphate at pH 10.0, and satisfactory results were obtained due to the improvement of peak shape and enhancement of separation (Fig. 2C). However, the analytical times were still longer because the EOF decreased with the addition of organic modifiers.

Direct UV absorbance detection at 270 nm was chosen because ICA, ICT, and DICT absorbs reasonably well at this wavelength. Moreover, no interference was observed at 270 nm. The applied voltage 12 kV and temperature 25 °C were achieved suitable baseline separation of ICA, ICT, DICT, and IS.

According to the factors mentioned above, the best resolution was obtained with 12 kV applied voltage and 25 °C column temperature; 30 mmol/L sodium tetraborate, 50 mmol/L monobasic sodium phosphate, and 50% acetonitrile at pH 10.0. A typical electropherogram of a mixture of three flavonoid standards and IS was shown in Fig. 3.

3.2. Calibration

Linearity, reproducibility, and recovery of the method were tested by analyzing standards of ICA prepared in blank serum.

The calibration curve of ICA in serum was linear in the range from 2.5 to 150 mg/L. The ratio of peak area was used for integration. The regression equation of calibration curve was $y = 0.0215x - 0.0314$. Its correlation coefficient ($r^2 = 0.9947$) showed a good linearity. This result showed the usefulness of the present CZE method following acetonitrile deproteinization in the assay of ICA from low to high serum levels. The minimum detectable concentration of ICA (signal-to-noise ratio of 3) in serum was determined to be, approximately, 1.0 mg/L.

To verify the precision of proposed CE method, the intra- and inter-day reproducibility of repeated assays were shown in Table 1. In the intra-day assay, the relative standard deviation (R.S.D.) for relative peak area ranges from 2.53 to 4.47%. In the inter-day assay, these values were 4.28 and 5.22%. These results suggest that separation and quantification with this system are reproducible for the measurement of ICA levels in rat serum.

The accuracy of the method was examined by extraction recovery studies conducted as described in Section 2.5.3. The recoveries of ICA were 69.3% ($n = 5$, 2.5 mg/L), 69.5% ($n = 5$,

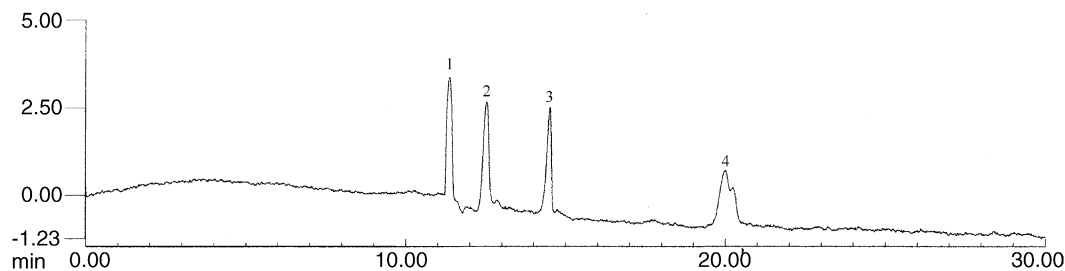

Fig. 3. A typical electropherogram of a blank serum spiked with icariin, icaritin, and desmethylicaritin. (1) Internal standard; (2) icariin; (3) desmethylicaritin; and (4) icaritin.

Table 1
Reproducibility of mean relative peak area of icariin

Concentration (mg/L)	Relative peak area ($\bar{x}$)	S.D.	R.S.D. (%)
Intra-day assay ($n = 5$)			
2.5	0.104	0.005	4.47
10	0.219	0.008	3.75
50	0.882	0.037	4.18
150	3.255	0.082	2.53
Inter-day assay ($n = 5$)			
50	0.847	0.044	5.22
150	3.237	0.138	4.28

10 mg/L), 74.2% ($n = 5$, 50 mg/L), 74.2% ($n = 5$, 100 mg/L), 77.2% ($n = 5$, 150 mg/L).

3.3. Determination of icariin in serum

A serum sample from a ICA-treated rat was shown in Fig. 4. The relative position of ICA in the electropherograms was in accordance with that of the standard solution.

Two metabolites were observed 30 min after administration by CZE including products 5 and 6 (Fig. 4). The compound under peak 5 has the similar migration time with DICT been identified as the possible demethylation product. However, the fact that ICT was not observed by CZE does not necessarily indicate that this metabolite was not formed. Biotransformation products not observed may be due to detection limitations.

3.4. Pharmacokinetic studies

The method proposed was successfully applied for pharmacokinetic studies of ICA in vivo. The pharmacokinetic parameters describing ICA disposition in the rats were presented in Table 2. The results indicated that a one-compartment was fitted to the data from individual rats (Fig. 5).

Table 2
Pharmacokinetic parameters of icariin after administration (ig) to four rats

Pharmacokinetic parameters	$\bar{x} \pm$ S.D.
K_e (min^{-1})	0.0117 ± 0.0027
$T_{1/2}$ (min)	59.18 ± 8.26
T_{max} (min)	51.81 ± 2.67
C_{max} (mg/L)	13.22 ± 1.02
$AUC_{0\rightarrow\infty}$ (mg min/L)	2471.1 ± 269.6
Cl/F (L kg^{-1} min^{-1})	0.0495 ± 0.0132
V_d/F (L kg^{-1})	4.36 ± 0.65

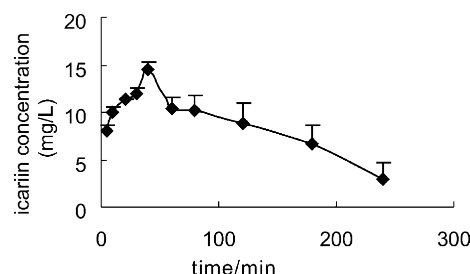

Fig. 5. Serum concentration of icariin–time curve after a single dose oral administration of 120 mg/kg icariin to rats ($n = 4$).

$AUC_{0\rightarrow\infty}$ of ICA was 2471.1 ± 269.6 mg min/L, which differed significantly from a previous report [19] that showed only1240 ± 348.1 mg min/L (a single dose of 100 mg ICA/kg was administered by gavage to Wistar rats). There are little differences in other pharmacokinetic parameters. The discrepancy between the two studies may be due to the different dosage taken. However, this difference also may be indicative of species differences in ICA disposition.

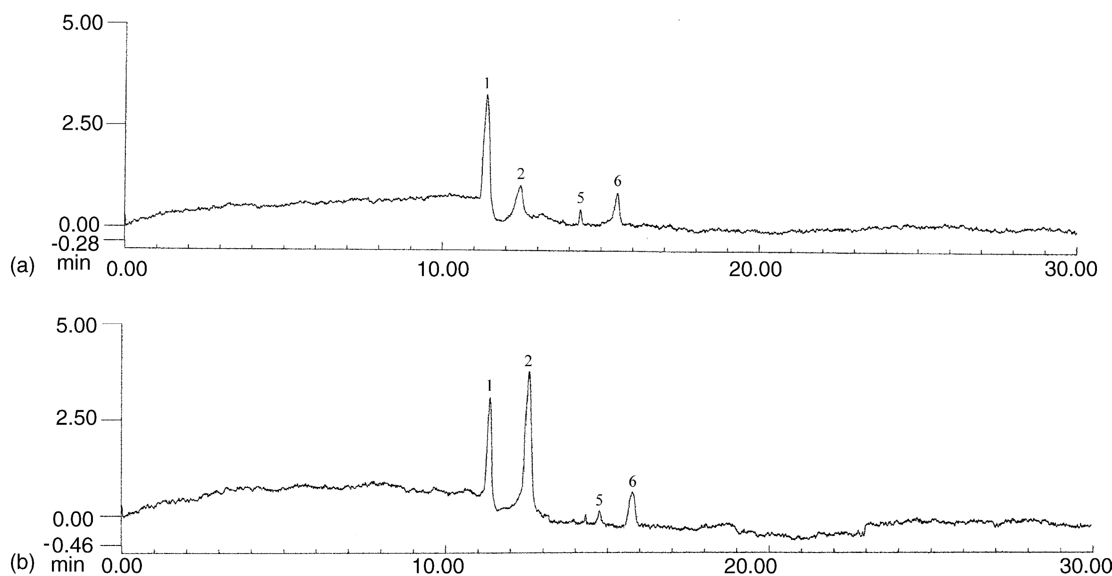

Fig. 4. Typical electropherograms of a serum sample from a rat treated with 120 mg icariin/kg ig (A), and addition of icariin to serum sample of a icariin-treated rat (B). (1) Internal standard; (2) icariin; and (5, 6) metabolites.

4. Conclusions

In summary, a CZE method was developed for the quantitation of ICA in rat serum, and was applied successfully in the determination of ICA-derived metabolites and evaluation of ICA pharmacokinetics in rats. This study showed that CZE is a reliable alternative approach for flavonoid compounds pharmacokinetic analysis. The described method can be extended for measuring other analogous substances in serum too.

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