Determination of three bile acids in artificial *Calculus Bovis* and its medicinal preparations by micellar electrokinetic capillary electrophoresis

Zhen Hu [a], Lang-Chong He [a,*], Jian Zhang [a], Guo-An Luo [b]

[a] *School of Medicine, Xi'an Jiaotong University, No. 205, ZhuQue Street, Xi'an, Shannxi 710061, China*
[b] *Department of Chemistry, Tsinghua University, Beijing 100084, China*

Abstract

A micellar electrokinetic capillary electrophoresis (MEKCE) method for the determination of cholic acid (CA), hyodeoxycholic acid (HDCA) and chenodeoxycholic acid (CDCA) in artificial *Calculus Bovis* and its four medicinal preparations is described. The buffer solution consisted of 40 mM disodic phosphate and 40 mM sodium dodecylsulfate (SDS) adjusted to pH 9.0. UV detection was set to 200 nm. Under optimum conditions, the analytes were baseline separated within 11 min. The linear calibration range was 12.1–970 μg ml^{-1} for CA and 18.8–950 μg ml^{-1} for HDCA and CDCA, respectively. It was found that overall recoveries were within the range of 98–102%, and R.S.D.s were less than 5% for the analytes. This method, due to its convenience, high accuracy and good reproducibility can be employed in quality control of artificial *Calculus Bovis* and its medicinal preparations.
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Keywords: Artificial *Calculus Bovis*; Capillary electrophoresis; Bile acids

1. Introduction

Calculus Bovis (known as cow bezoar, a type of rare Chinese medicinal material) was first recorded in 'Shennong Bencao Jing' and has been used clinically in China for 2000 years [1]. It is the dry gallstone of *Bos taurus domesticus* Gmelin and, according to the theory of traditional Chinese medicine, has the effect of sedation, anti-hyperspasmia, relieving fever, diminishing inflammation and normalizing function of the gallbladder [2]. Because of limited source of natural *Calculus Bovis*, artificial *Calculus Bovis* is always applied as a replacer in the medicinal preparations. Artificial *Calculus Bovis* has two kinds of active components—bilirubin and bile acids [3]. Bile acids are a mixture of steroids, which mainly include cholic acid (CA), hyodeoxycholic acid (HDCA), chenodeoxycholic acid (CDCA), among others [4] (chemical structures are shown in Fig. 1). Spectrophotometry [5,6], thin

layer chromatography (TLC) [7] and high-performance liquid chromatography (HPLC) [8–10] are commonly employed to determine the amounts of bile acid in raw materials, Chinese traditional medicine preparations [11] and other biosamples [12]. However, using these methods, a derivatization process of the samples is usually needed, making the results of determinations more uncertain than they are when this process is omitted. High-performance liquid chromatography-evaporative light scattering detector (HPLC–ELSD) method can be used in the underivatized case, but its sensitivity is well known to be unsatisfactory and the running time to be longer [10].

Capillary electrophoresis (CE), because of its high resolution and sensitivity, minimal sample volume, low time-consumption and high separation efficiency [13] is now a widely employed separation technique, and has been successful in pharmaceutical analysis [14–17], especially in the analysis of traditional Chinese medicine [18–20]. A micellar electrokinetic chromatography method (MEKC) has been used for the determination of dehydrocholic acid (DHCA) and ursodeoxycholic acid (UDCA) [21] and for deoxycholic acid (DCA) [22]. However, there are

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* Corresponding author. Tel.: +86 29 8265 5142; fax: +86 29 8265 5451.
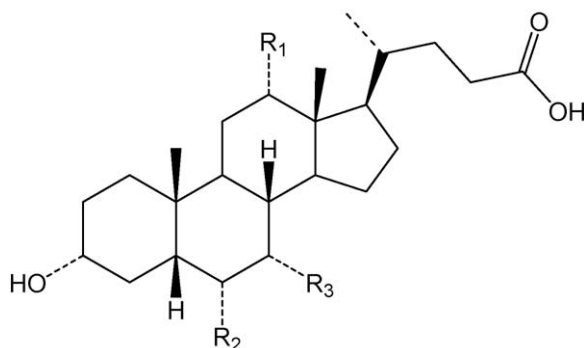*E-mail address:* helc@mail.xjtu.edu.cn (L.-C. He).

Fig. 1. Chemical structures of the major bile acids found in artificial *Calculus Bovis*. CA: $R_1 = -OH = R_2 = R_3$; CDCA: $R_2 = -OH$: $R_1 = R_3 = -H$; and HDCA: $R_3 = -OH$, $R_1 = R_2 = -H$.

few reports on the analysis of bile acids in a complex system, such as traditional Chinese medicine. In this study, we developed a sensitive and reliable method (capillary electrophoresis–diode array detector, CE–DAD) for the simultaneous determination of three bile acids in the raw material, i.e. artificial *Calculus Bovis*, and its four medicinal preparations.

2. Experimental

2.1. Materials and reagents

Cholic acid, hyodeoxycholic acid, chenodeoxycholic acid, disodium phosphate and sodium dodecylsulfate (SDS) were obtained from Sigma (St. Louis, USA). Methanol and acetonitrile were HPLC grade and supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained from Hitech-Kflow™ RO Equipment (Shanghai, China). All other reagents were of analytical grade. Solutions and samples were filtered through a 0.45 mm nylon membrane (Micron Separations Inc., Westboro, MA, USA) and degassed before use.

The raw material of artifical *Calculus Bovis* was purchased from Shaanxi Herbals Corp., and the place of production was Shaanxi Province. Niuhuang Jiedu pills (NJP, Yabao Parmaceutical Group, Shanxi province, lot 40630), Niuhuang Qinggan capsules (NQC, Aolida-Tede Parmaceutical Corp., Heilongjiang province, lot 31001), Niuhuang Xiaoyan tablets (NXT, HRB Huaya Medicined Group Corp., Heilongjiang province, lot 20040805) and Qingkailing injection (QI, Shineway Parmaceutical Corp., Hebei province, lot 051006) were all purchased from Shaanxi Baixing Dispensary.

2.2. Apparatus

Micellar electrokinetic capillary electrophoresis (MEKCE) was carried out with a G1600AX CE system coupled with a diode array detector (Agilent Technologies, Waldbronn, Germany). Data were collected and processed by ChemStation™ software (Agilent Technologies). An uncoated fused-silica capillary of 70 cm length (58 cm to the detector) and 75 μm I.D. (Agilent Technologies) was used.

2.3. CE conditions

The analytical procedure by MEKCE was performed by employing a running buffer consisting of 40 mM disodic phosphate buffer, pH 9.0 (adjusted with sodium hydroxide), with 40 mM SDS. Samples were injected by applying a pressure of 0.5 psi for 5 s, an operating voltage of 20 kV at a temperature of $25 \pm 0.1\,^{\circ}$C and UV detection at 200 nm.

At the beginning and end of each day, the capillary was rinsed with 0.1 M sodium hydroxide for 5 min, then washed with water for 10 min and finally with background electrolyte for 15 min. Between runs, the capillary was flushed with running electrolyte for 3 min.

2.4. Stock and working solutions

Stock solutions of CA, CDCA and HDCA in methanol containing 1.0 mg ml^{-1} were prepared. Stock solutions were diluted by adding 40 mM disodic phosphate buffer to provide a series of working solutions, each of which was injected directly in triplicate, and the corrected peak area was used for the calibration curve.

2.5. Sample preparation

A 0.1 g sample of dried artificial *Calculus Bovis* powder was accurately weighed in a 50.0 ml volumetric flask and mixed with 40 ml of chloroform. The mixture was stirred in an ultrasonic bath for 30 min, and then topped up to 50.0 ml with chloroform. Then, 30 ml of the suspension was centrifuged at 5000 rpm for 10 min and a 25 ml aliquot of supernatant solution was evaporated to dryness. The residue was dissolved using the running buffer and topped up to 10.0 ml in a volumetric flask.

A total of 10 pills of NJP, 50 tablets of NXT and 50 capsules of NQC were weighed accurately, and the average weights were calculated. Then, the samples were finely powdered and an amount equivalent to one pill for NJP, five tablets for NXT and five capsules for NQC were accurately weighed. The samples of the solid doses were further prepared according to the same process under which the dried powder of artificial *Calculus Bovis* was handled.

A total of 10 ampoules of QI (10 ml per flask) were mixed and 20.0 ml was extracted twice with 25 ml chloroform. The extracts were combined and evaporated to dryness; the residue was dissovled using the running buffer and topped up to 10.0 ml in a volumetric flask.

Then each of the sample solutions was filtered first through filter paper and then through a 0.22 μm syringe filter. After filtration, the solutions were injected directly into the CE system for analysis. All sample solutions were stored in the dark until use.

3. Results and discussion

To achieve satisfactory separation, capillary zone electrophoresis (CZE) and micellar electrokinetic capillary electrophoresis methods were compared firstly. Then, the effects of running electrolyte concentration, SDS concentration, pH of

running buffer and applied voltage on migration time were investigated.

3.1. Comparison between CZE and MEKCE

In view of chemical structures of the three bile acids (Fig. 1), it seems that these compounds should be separated by CZE using alkaline electrolyte because all compounds have carboxyl groups. However, from preliminary tests, the compounds could not be separated satisfactorily, no matter how the CZE conditions were varied. It is well known that the migration velocity of solute in a capillary (v_α) is the vectorial sum of electrophoresis (v_{ep}) and electroosmotic flow (v_{EOF}) velocities. Then, it is clear that under the same conditions of the CE system, v_{EOF} could be considered as a constant and v_{ep} will directly determine v_α. Thus, formal charge and Stoke's radius of the solute are the main factors influencing separation. In this study, the formal charge of the three analytes are the same, while Stoke's radius are almost the same. Therefore, the compounds could scarcely be separated by a simple CZE system, and the MEKCE system was implemented to analyze these compounds in the samples.

3.2. Effect of SDS concentration

Concentrations of SDS ranging from 30 to 100 mM were employed in conjunction with 40 mM disodic phosphate at pH 9.0. The results showed that the concentration of SDS surfactant is very important in the separation of the bile acids. Fig. 2 summarized the effects of SDS concentration on the separations. It was found that the migration time increased as SDS concentration increased. The three compounds were almost baseline separated from the other components at 50 mM. However, the migration time shortened with increasing SDS concentration over 50 mM because of the influence of the current across the
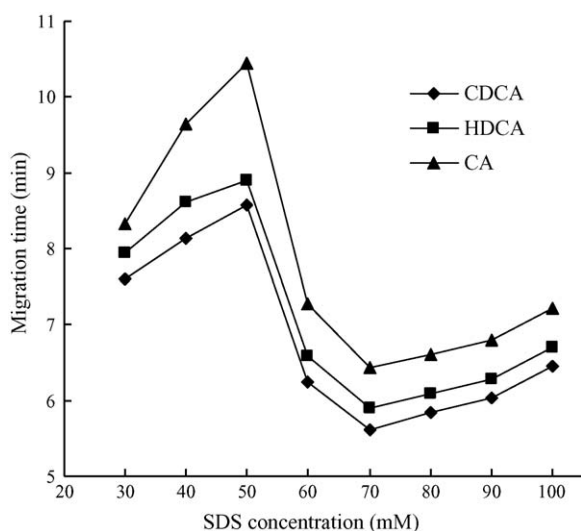
Fig. 2. Effect of SDS concentration on migration time of analytes. Capillary: 70 cm (28 cm to the detector) × 75 μm I.D. Buffer: 40 mM disodic phosphate at pH 9.0. Voltage 20 kV. Detection 200 nm. Pressure injection 5 s. Capillary temperature 25 °C. The migration time of each point in the figure is the average value of three runnings, and the R.S.D. are all less than 1%.
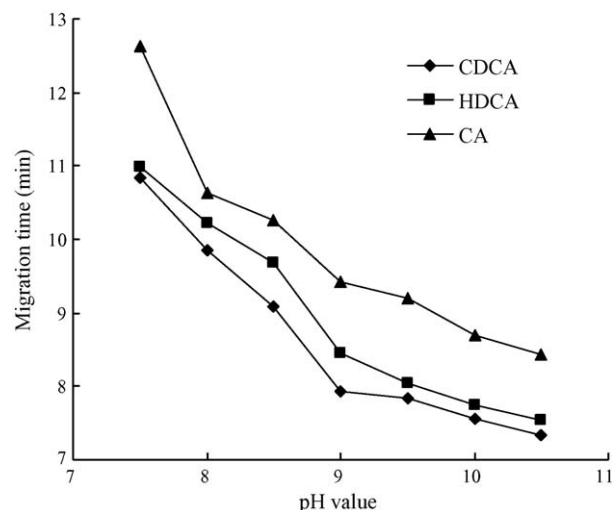
Fig. 3. Effect of buffer pH values on migration time of analytes. Other conditions as in Fig. 2. The migration time of each point in the figure is the average value of three runnings, and the R.S.D. are all less than 1%.

capillary, and because the resolution of the compounds was decreased. In fact, there was a little better resolution between HDCA and CDCA at 40 mM SDS than that at 50 mM SDS. So that, 40 mM was selected as the optimal concentration of SDS.

3.3. Effect of buffer pH

The acidity of the electrophoretic medium is also a governing factor in the separation of ionizable analytes because it determined the extent of ionization of each individual analyte. To verify the effect of pH on migration behavior of the analytes, experiments were performed with 40 mM disodic phosphate and 40 mM SDS as buffer. As shown in Fig. 3, the migration time of the three analytes decreased in the investigated range (from 7.5 to 10.5). When the pH was <8.0, the three compounds could not be separated completely, but resolution improved when pH was in the range 8.5–9.5. Baseline separation was achieved at pH 9.0 but then resolution decreased when pH was >9.5, which may have been due to the increased ionic strength of the buffer, leading to a higher production of Joule heat. From the above findings, pH 9.0 was selected as the optimum pH for the separation.

3.4. Effect of concentration of disodic phosphate

Buffer concentration markedly affected the separation because it influenced the EOF and viscosity of the electrolyte. To obtain the best resolution of the three analytes, the effect of the concentration of disodic phosphate was investigated in the range of 10–60 mM, with 40 mM SDS, at pH 9.0. The migration time of the three analytes increased with increasing disodic phosphate concentration. As Fig. 4 indicates, when disodic phosphate concentration was less than 35 mM, a baseline separation for HDCA and CDCA was not obtained. Migration time increased with increased disodic phosphate concentration. The resolution could be increased slightly when the disodic phosphate concen-
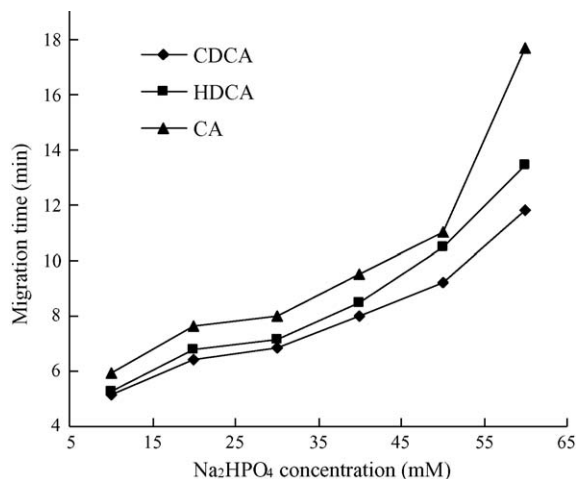
Fig. 4. Effect of buffer concentration on migration time of analytes. Other conditions as in Fig. 2. The migration time of each point in the figure is the average value of three runnings, and the R.S.D. are all less than 1%.

tration was greater than 50 mM, but the analysis time was more prolonged. The detection sensitivity and resolution were optimal when disodic phosphate concentration was 40 mM. Therefore, the optimum concentration of disodic phosphate was 40 mM.

3.5. Effects of other factors

To understand the other factors that influenced the separation, organic modifiers, running voltage and capillary temperature were also investigated. It was found that: (1) no significant improvement in separation was obtained when either methanol or acetonitrile was employed in various concentrations as an organic modifier; (2) with increasing voltage, the migration time and resolution of each compound decreased; and (3) with increasing capillary temperature, the migration time curves of the three analytes decreased in parallel. After that, no organic modifiers were used in the separation, and the separation was achieved by using a capillary temperature of 25 °C and a 20 kV running voltage, combined with the other optimum conditions mentioned above. Typical electrophoregrams of standards and samples are illustrated in Fig. 5.
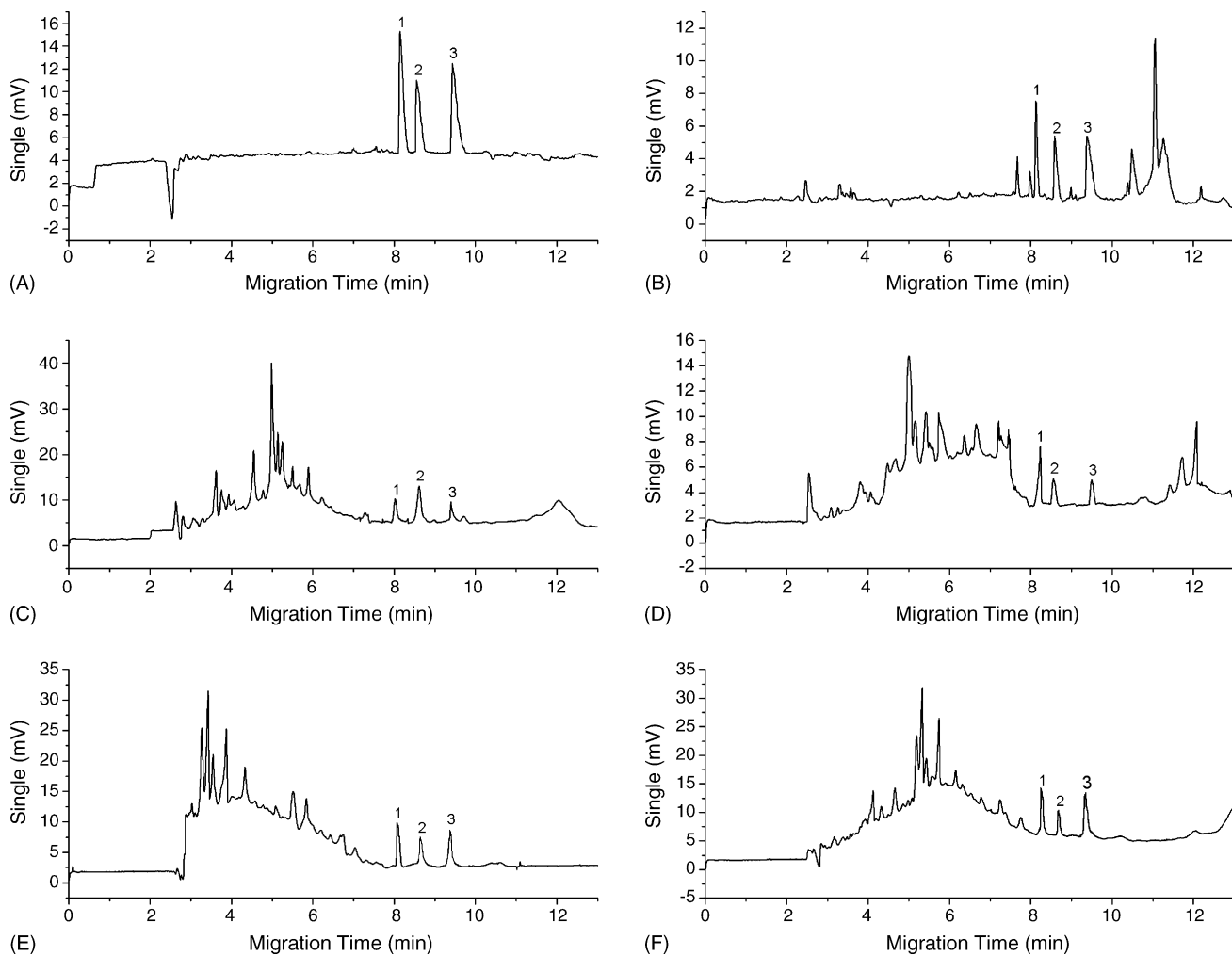

Fig. 5. Electrophoregrams of: (A) standard solutions containing 1, CDCA 238 $\mu g\,ml^{-1}$; 2, HDCA 238 $\mu g\,ml^{-1}$ and 3, CA 243 $\mu g\,ml^{-1}$; (B) *Calculus Bovis* sample solution; (C) NJP sample solution; (D) QI sample solution; (E) NXP sample solution; and (F) NQC sample solution. Peaks: (1) CDCA; (2) HDCA; and (3) CA. Experimental conditions as given in the text.

Table 1
Electrophoretic system precision

Component	Peak area/tm (R.S.D. %)		tm (R.S.D. %)	
	Intra-day assay ($n=6$)	Inter-day assay[a] ($n=18$)	Intra-day assay ($n=6$)	Inter-day assay* ($n=18$)
CA	0.99	1.41	0.80	1.10
HDCA	0.95	1.53	0.96	1.25
CDCA	1.10	1.31	0.98	1.09

[a] Mean values obtained on 3 different days from replicate injections ($n=6$) of a standard solution.

Table 2
Linearity and detection limits

Linearity	CA	HDCA	CDCA
Parameters			
Concentration range studied ($\mu g\,ml^{-1}$)	12.1–970	11.8–950	11.8–950
Number of concentration level	5	5	5
Regression results			
Correlation coefficient (r^2)	0.9990	0.9992	0.9991
Intercept	5.3660	2.2644	1.5891
Slope	0.0802	0.0799	0.0874
R.S.D. at different concentration levels (%)	0.25–0.55	0.26–0.60	0.26–0.58
LQD ($\mu g\,ml^{-1}$)	12.1	11.8	11.8
LOD ($\mu g\,ml^{-1}$)	3.02	2.98	2.98

Table 3
Stability of the samples ($n=6$)

Sample	CA (R.S.D. %)		HDCA (R.S.D. %)		CDCA (R.S.D. %)	
	Peak area/tm	tm	Peak area/tm	tm	Peak area/tm	tm
Artificial *Calculus Bovis*	1.51	1.23	2.01	2.03	1.30	1.75
NJP	1.82	1.65	2.02	1.91	1.66	1.80
QI	1.67	1.85	1.97	1.98	1.67	1.79
NXT	1.88	1.54	1.75	1.87	1.54	1.70
NQC	1.76	1.83	1.98	2.01	1.46	1.57

3.6. Validation of the method

In the validation of the method, analytical figures of merit, such as selectivity, linearity, range, limits of detection and quantitation, accuracy and precision were evaluated.

Selectivity was demonstrated by examining migration times, by spiking of standard solutions to the real samples, confirming the absence of interference with analyte peaks and estimating resolution factors. By this method, there was no interference with the analyte peaks, and the theoretical plates of the three peaks were all greater than 10,000, asymmetries were between 0.95 and 1.05 and all resolutions exceeded 1.5. These showed good selectivity of the method for the analytes.

Repeatability and intermediate precision of the electrophoretic system were evaluated for migration times and normalized peak areas. Data are summarized in Table 1.

Linear calibration curves were obtained for each component at five different concentration levels, over the range investigated. Each solution was injected twice. The correlation coefficients were calculated by linear regression analysis equations. Data obtained are summarized in Table 2.

The lowest limit of the range was defined as the limit of quantitation (LOQ) level, and the limit of detection (LOD) was

Table 4
Determination results of recovery using this method ($n=3$)

Level	Mean recovery (%), R.S.D. (%)		
	50	100	150
Artificial *Calculus Bovis*			
CA	110.4, 5.36	101.2, 3.21	101.4, 3.47
HDCA	105.8, 4.73	98.1, 3.44	100.1, 3.21
CDCA	95.4, 5.16	103.2, 3.11	99.8, 4.21
NJP			
CA	90.6, 5.23	101.4, 4.31	98.5, 3.55
HDCA	96.7, 4.78	99.4, 3.59	99.7, 3.45
CDCA	102.4, 5.16	102.41, 4.01	100.2, 4.00
QI			
CA	86.4, 4.21	98.8, 3.33	97.6, 4.21
HDCA	93.7, 3.15	99.6, 2.78	99.1, 3.40
CDCA	96.5, 3.21	101.4, 3.11	98.7, 3.36
NXT			
CA	89.7, 3.28	102.4, 2.12	98.6, 4.32
HDCA	96.6, 4.10	99.5, 3.29	99.5, 4.10
CDCA	97.8, 3.65	98.9, 2.98	101.1, 3.98
NQC			
CA	96.1, 4.31	101.4, 3.28	97.9, 4.00
HDCA	112.2, 3.78	99.8, 3.14	99.1, 3.76
CDCA	101.4, 4.17	103.4, 4.21	97.2, 3.61

Table 5
Contents of the analytes in the samples

Component	Laboratory	Amount (%), R.S.D.$_1$[a] (%) ($n = 5$)				
		Artificial *Calculus Bovis*	NJP	QI[b]	NXT	NQC
CA	1	4.23, 2.10	2.56, 3.03	20.3, 2.11	0.51, 2.01	0.48, 3.11
	2	4.29, 2.35	2.40, 2.55	20.6, 3.01	0.46, 3.07	0.50, 2.87
	3	4.04, 2.05	2.45, 2.74	19.4, 2.87	0.49, 3.14	0.53, 2.80
R.S.D.$_2$[c] (%)		3.12	3.31	3.11	5.17	4.99
HDCA	1	3.71, 3.01	1.53, 2.74	11.2, 3.12	0.20, 3.09	0.20, 2.73
	2	3.51, 2.45	1.44, 3.01	10.8, 2.57	0.19, 3.11	0.21, 2.85
	3	3.69, 3.11	1.51, 2.98	10.6, 3.03	0.18, 2.78	0.22, 3.12
R.S.D.$_2$ (%)		3.03	3.16	2.81	5.26	4.76
CDCA	1	4.18, 2.95	1.47, 2.91	12.2, 2.89	0.27, 2.91	0.25, 2.31
	2	4.23, 2.74	1.43, 2.77	12.0, 3.00	0.30, 2.88	0.26, 2.74
	3	4.01, 3.01	1.50, 2.69	11.5, 2.69	0.29, 2.78	0.24, 2.91
R.S.D.$_2$ (%)		2.79	3.41	3.03	5.33	4.00

[a] R.S.D.$_1$ is intra-laboratory R.S.D.
[b] Unit of amount of QI is $\mu g \, ml^{-1}$.
[c] R.S.D.$_2$ is inter-laboratory R.S.D.

estimated by diluting a test sample solution until the signal-to-noise ratio reached approximately three. The results shown in Table 2. R.S.D. values ($n = 6$) for LOQ were 4.5% for CA, 5.5% for HDCA and 5.1% for CDCA.

When average migration times ($n = 6$) of standard solutions were compared to those of the spiked blank sample, no significant statistical difference was observed ($P < 0.05$), showing no matrix effect and, therefore, external calibration standards proved to be suitable for quantitation of these components.

Stability of the samples was investigated by examining normalized peak areas and migration times of the analytes in artificial *Calculus Bovis* and the four preparations at 0, 6, 12, 24, 36 and 48 h. The data were expressed as R.S.D. values in Table 3.

The accuracy of the CE method was assessed over the same concentration range that was investigated in the linearity study (50–150%) by making three independent determinations at each concentration level, and the results obtained are displayed in Table 4.

Robustness was investigated by modification of the background electrolyte with variations of 95% in pH values, and of 95% in SDS and buffer concentrations. No significant effect on resolution between the nearest peaks was observed.

3.7. Sample analysis

The method was employed for the analysis of CA, HDCA, and CDCA in crude potions of artificial *Calculus Bovis*, and four related preparations. Typical electrophoregrams of the samples are illustrated in Fig. 5. The peaks were identified by comparing the migration times and by spiking the standards to the sample solution. The contents of the three compounds in the five samples are given in Table 5. The results suggest that the three components could be more conveniently monitored in artificial *Calculus Bovis* and its related preparations.

4. Conclusions

The results demonstrate that MEKCE, as proposed in this work is simple, rapid, sensitive and universal for the determination of CA, HDCA and CDCA in artificial *Calculus Bovis* and its medicinal preparations, and valuable for the quality control of traditional Chinese medicinal preparations. The proposed MEKCE method is not only a good alternative for simultaneous analysis of these active components but also a promising supplementary technique for the preliminary investigation of other bile acids in other 'herbs' and related medicinal preparations. The results presented here can hopefully further advance the current knowledge of Chinese traditional medicine.

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