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Carboxymethylchitosan covalently modified capillary column for open tubular capillary electrochromatography of basic proteins and opium alkaloids

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ABSTRACT

A novel open tubular (OT) column covalently modified with hydrophilic polysaccharide, carboxymethylchitosan (CMC) as stationary phase has been developed, and employed for the separations of basic proteins and opium alkaloids by capillary electrochromatography (CEC). With the procedures including the silanization of 3-aminopropyltrimethoxysilane (APTS) and the combination of glutaraldehyde with amino-silylated silica surface and CMC, CMC was covalently bonded on the capillary inner wall and exhibited a remarkable tolerance and chemical stability against 0.1 mol/L HCl, 0.1 mol/L NaOH or some organic solvents. By varying the pH values of running buffer, a cathodic or anodic EOF could be gained in CMC modified column. With anodic EOF mode (pH < 4.3), favorable separations of basic proteins (trypsin, ribonuclease A, lysozyme and cytochrome C) were successfully achieved with high column efficiencies ranging from 97,000 to 182,000 plates/m, and the undesired adsorptions of basic proteins on the inter-wall of capillary could be avoided. Good repeatability was gained with RSD of the migration time less than 1.3% for run-to-run ($n = 5$) and less than 3.2% for day-to-day ($n = 3$), RSD of peak area was less than 5.6% for run-to-run ($n = 5$) and less than 8.8% for day-to-day ($n = 3$). With cathodic EOF mode (pH > 4.3), four opium alkaloids were also baseline separated in phosphate buffer (50 mmol/L, pH 6.0) with column efficiencies ranging from 92,000 to 132,000 plates/m. CMC-bonded OT capillary column might be used as an alternative medium for the further analysis of basic proteins and alkaline analytes.

1. Introduction

Open tubular capillary electrochromatography (OTCEC) is a very promising analytical separation technique owing to simple column preparation, no bubble formation and stable EOF application, and has gained increased attention in recent years [1,2]. In open tubular CEC, the stationary phase is tethered or coated to the capillary inner wall [3,4], it does not require the fabrication of any frits for packed formats or blending of monomeric reagents with suitable porogens in precise proportions for monoliths [5,6]. OT column can confer high efficiency due to a flat flow induced by electroosmosis, and has been playing an important role in the rapid separations of chiral molecules [7,8], proteins [9,10] and environmental compounds [11]. The preparation of stationary phase is critical for OTCEC. Development of new modified OT columns and its application are receiving more and more attention.

Many coating techniques have been used to develop OT columns, such as chemical modified covalent coating [12,13], physically adsorbed coating [14,15], and dynamic coating [16]. Covalent modification with long-term stability and flexible manipulation might be a favorable choice [17,18], and the development of new coatings for OTCEC still remains as an active area of research [19–21]. Polysaccharides and their derivatives, which possess of good hydrophilicity, have attracted much attention to modify the capillary column [22–25]. Chitosan (CS), a kind of saccharide, has an ideal biocompatibility, good film-forming properties and has been considered as one of the potential chromatographic matrix [26–28]. Recently, chitosan has been employed as a dynamic and static cationic adsorbed coating for the analysis of basic proteins [29]. Although satisfactory separations for some proteins have been achieved, a limited pH range of 3.0–5.5 was used in the work. At the same time, the stability of chitosan coating needed to be improved. In Huang's work [30], an improved chitosan coating was developed to overcome these problems, chitosan was first adsorbed to the capillary wall, then cross-linked with glutaraldehyde, which coated

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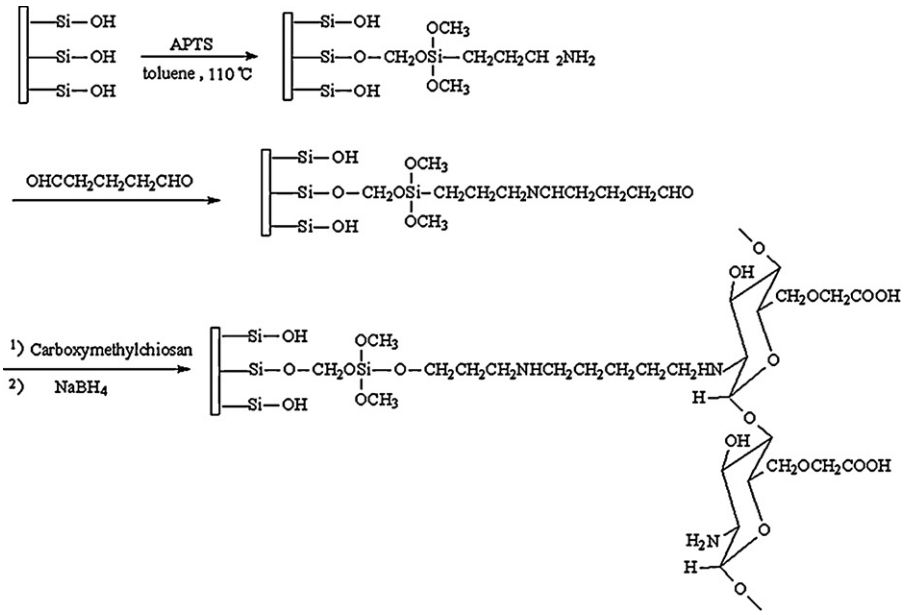

Fig. 1. . Schematic of the immobilization of CMC onto the inner surface of fused silica capillary.

the capillary wall like a "carpet". However, the CS coating changed obviously after being rinsed with the solution of 0.1 mol/L NaOH or 0.1 mol/L HCl.

Carboxymethylchitosan (CMC) is a chitosan derivative. It has better solubility in water than chitosan. Its good water-solubility, non-cytotoxicity and good bioactivity make CMC an important derivative. To date, few studies on CMC for modified coating on inner wall of capillary have been reported. Fu et al. [31] utilized CMC as a new capillary modified reagent by physical adsorption and applied it for the CE separation of proteins, and forecasted that it could be widely applied to analyse of biomolecules. However, it was deficient in the tolerance to the medium with too low or too high pH values.

In this study, through the covalent coupling with spacer arm glutaraldehyde, a functional OT column modified with CMC was obtained. Due to free amino groups on its structural unit, CMC could be grafted to amino-silylated capillary surface from the side-chain, and a permanent CMC-coated capillary column by covalently bonding was achieved and observed by SEM. The capability of CMC coating column for the separation of basic proteins and alkaloids was subsequently evaluated. It was found that the CMC modified column could be successfully applied to the OTCEC analysis of basic proteins (including trypsin, ribonuclease A, lysozyme and cytochrome C) and opium alkaloids with satisfied stability and good repeatability.

2. Experimental

2.1. Chemicals and materials

Carboxymethylchitosan (CMC, average molecular mass ca. 300,000, degree of deacetylation 85%, and degree of substitution is 80%) was obtained from Dalin Xindie Chitin Co., Ltd. (Dalin, China). Morphine, thebaine, narcotine and papaverine were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ribonuclease A (from bovine pancreas) and trypsin (from bovine pancreas) were purchased from Sigma (St. Louis, MO, USA), lysozyme (from egg white) and cytochrome C (from horse heart) were purchased from Biodee Biotechnolody Co., Ltd. (Beijing, PR China). 3-Aminopropyltrimethoxysilane (APTS) was obtained from Sigma–Aldrich. Glutaraldehyde (25% water solution) and the other reagents were obtained from Shanghai Chemical Reagents Co. (Shanghai, China). All chemicals used were of analytical or chro-

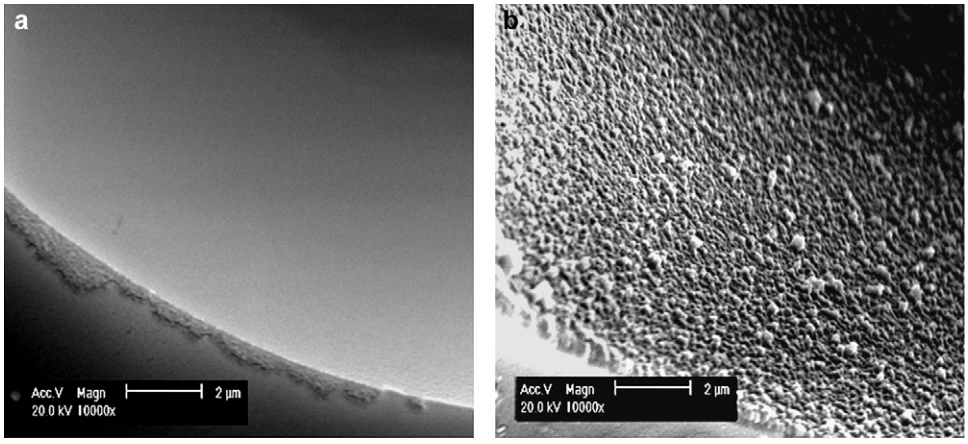

Fig. 2. . SEM photographs of (a) bare silica capillary and (b) CMC modified capillary inner surface.

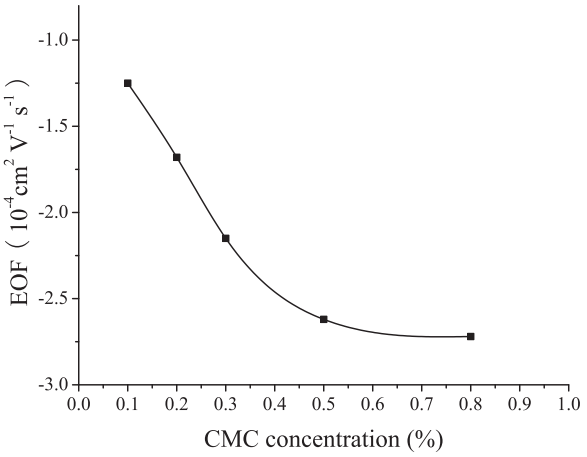

Fig. 3. . Effect of CMC concentration on EOF. Conditions: column: CMC-modified capillary column: 57.0 cm total length (37.0 cm effective length) × 50 μm i.d.; mobile phase: 20 mM phosphate buffer at pH 3.0; applied voltage, +15 kV; wavelength: 214 nm.

matographic grade. Fused silica capillary (375 μm o.d. × 50 μm i.d.) was obtained from Yongnian Ruifeng Instrumental Co. (Hebei, China).

2.2. Sample preparation

Carboxymethylchitosan stock solution, 1% (w/v) aqueous solution was prepared by stirring at room temperature then filtering through sand core filter. Opium alkaloids were dissolved in methanol and then diluted at appropriate concentration with buffer solution. Protein sample solutions were prepared by dissolving sample compounds in deionized water to form the stock solutions with the concentrations of 1 mg/mL, followed by diluting corresponding stock solutions with an appropriate amount of buffer solution.

2.3. Apparatus and analytical procedures

The experiments were performed on a Trisep 2010 GV CEC system (Unimicro Technologies, Pleasanton, CA, USA) equipped with a UV/Vis detector. Data collection and instrument controlling were through software of HW-2000 chromatography data handling system (Qianpu Software Ltd., Nanking, China). Sample injections were performed electronkinetically for 3 s at 8 kV. Toluene was used for an electroosmotic flow (EOF) marker. Phosphate buffers were prepared for mobile phase. Before CEC experiments, the column was conditioned with the mobile phase for 0.5 h. The column was equilibrated for 15 min when the mobile phase was changed. When proteins were analyzed, the column was rinsed with the mobile phase for 5 min between run and run. Then, the column was equilibrated electrokinetically at the operating voltage until the baseline and current were stable. Scanning electron micrography (SEM) picture of the capillary were taken on a XL30 E scanning electron microscope (Philips, Netherlands).

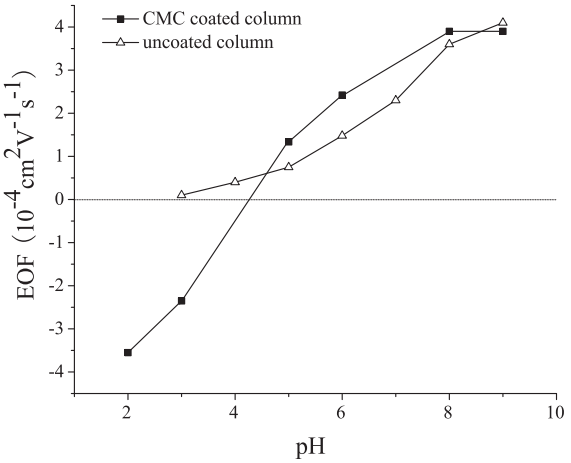

Fig. 4. . Comparison of EOF on uncoated capillary and CMC-modified capillary. Conditions: columns: 60.0 cm total length (40.0 cm effective length) × 50 μm i.d.; mobile phase: 20 mM phosphate buffer of various pH values; applied voltage, ± 15 kV; wavelength: 214 nm.

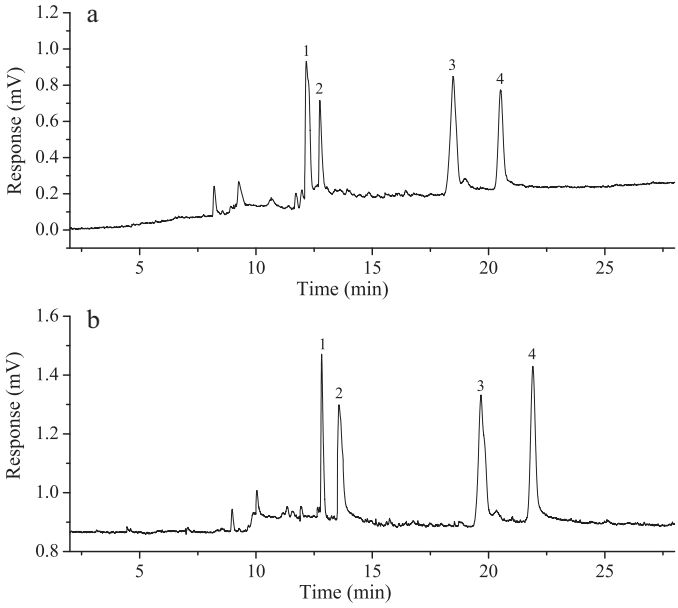

Fig. 5. Electrochromatogram of four proteins in CMC-modified column at (a) pH 2.5 and (b) pH 3.0. Conditions: column: 57.0 cm total length (37.0 cm effective length) × 50 μm i.d.; mobile phase: 20 mM phosphate buffer (pH 2.5 or pH 3.0); applied voltage: +15 kV; wavelength: 214 nm. Peaks: 1, trypsin; 2, ribonuclease A; 3, cytochrome C; 4, lysozyme.

2.4. Column preparation

Capillary was firstly pretreated by the procedure described in the literature [32]. Then the capillary was functionalized by filling with 3-aminopropyltrimethoxysilane (APTS) (30%, v/v) in toluene, and plugged with GC septa and reacted at 110 °C for 12 h. The silylated capillary was rinsed with toluene to remove unreacted material. 10% (v/v) Glutaraldehyde dissolved in 50 mmol/L phosphate buffer solution (pH 8.0) was applied to the capillary for 60 min and then the capillary was rinsed with water and methanol for 30 min respectively, followed by blowing with nitrogen for 1 h. Finally, the capillary was rinsed with carboxymethylchiosan solution for 20 min and contacted statically at 60 °C for 60 min, followed by blowing with nitrogen for 5 min and then rinsed with 10% (v/v) glutaraldehyde for 60 min, 10% sodium borohydride solutions (dissolved in buffer solution at pH 10) for 30 min and water for 30 min, respectively. The schematic of the immobilization of CMC onto the fused silica capillaries was shown in Fig. 1.

Table 1
Chemical tolerance against solvents of different modified columns.

Rinsing solvent	Rising time (min)	Variation ratio of EOF (%)[A]	Column	Reference
	15	12.25	Column 1: CS coating	[30]
0.1 mol/L NaOH	15	12.3	Column 2: CMC coating by physical absorption	[31]
	30	0.7	Column 3: CMC coating by covalent coupling	This paper
	15	3.64	Column 1	[30]
0.1 mol/L HCl	15	6.23	Column 2	[31]
	30	0.6	Column 3	This paper
	15	1.79	Column 1	[30]
CH_3OH	15	1.14	Column 2	[31]
	30	1.4	Column 3	This paper
	15	2.72	Column 1	[30]
CH_3CN	15	1.41	Column 2	[31]
	30	0.8	Column 3	This paper

[A] Variation ratio of EOF = $(EOF^b - EOF^a)/EOF^a \times 100$ (%). EOF^a and EOF^b was the EOF measured before or after rinsing with the solvent. Conditions: applied voltage, +15 kV; buffer, 20 mmol/L phosphate buffer at pH 3.0; capillary, 50 μm i.d.; 57.0 cm (37.0 cm effective length); wavelength: 214 nm.

Table 2
Reproducibility of migration time and column efficiency of tested proteins.

Protein	Molecular weight	p*I*	Efficiency (plate/m)	Reproducibility (% RSD)			
				Migration time		Peak area	
				Run-to-run ($n = 5$)	Day-to-day ($n = 3$)	Run-to-run ($n = 5$)	Day-to-day ($n = 3$)
Trypsin	23,700	8.7	110,000	0.7	1.4	3.8	6.8
Ribonuclease A	13,700	9.3	182,000	0.8	1.6	4.4	7.5
Cytochrome C	12,500	10.1	97,000	1.0	2.3	5.1	8.3
Lysozyme	14,300	11.2	127,000	1.3	3.2	5.6	8.8

Conditions: column: 57.0 cm total length (37.0 cm effective length) × 50 μm i.d.; mobile phase: 20 mM phosphate buffer at pH 3.0; applied voltage: +15 kV; wavelength: 214 nm.

3. Results and discussion

3.1. Characterization of CMC columns

3.1.1. SEM images

Morphology of the prepared capillary column modified with carboxymethylchitosan was observed by SEM and shown in Fig. 2b. Compared with the bare fused silica column (Fig. 2a), the morphology of a polymer layer tightly coated onto the prepared capillary column could be sighted. It was considered that both the phase ratio and the surface area of the polymer coated column were greatly increased as compared with the bare capillary column.

3.1.2. Effect of CMC concentration on the electroosmotic mobility

As shown in Fig. 3, CMC was positively charged, and an inverse electroosmotic flow (EOF) could be gained at pH 3.0. The value of EOF enhanced with the concentration of CMC increasing, which indicated that more CMC could be coated onto the inner wall. When the concentration of CMC ranged in 0.5–0.8% (w/v), the EOF rised slightly and reached the maximum value of 0.8%, which indicated a relative saturation of CMC coating onto the capillary surface was achieved. To gain the best polymer coating, the concentration of 0.8% was chosen for further research.

3.1.3. Electroosmotic mobility measurement

As shown in Fig. 4, the EOF of the uncoated capillary was in the direction from anode to cathode and increased with the rising of pH, which was due to the dissociation of silanol groups on the capillary wall. The EOF of uncoated column was almost suppressed below pH 3.0. In CMC-modified capillary, the EOF was in inversed direction when the pH of running buffer was below 4.3. The magnitude of EOF on OT column was ascribed to the net surface charge density of all of the ionizable groups. On the CMC modified column surface, there were amino groups and carboxylic groups on the structure of carboxymethylchitosan, as well as the remaining silanol groups on the capillary surface. When the pH of running buffer was below 4.3, the net surface charge of the column was mainly due to the ionized amino groups, while the dissociation of carboxylic groups and silanol groups were suppressed, so the column exhibited anodic EOF. When the pH value exceeded 4.3, the cathodic EOF was obtained. It indicated that the dissociation of the carboxylic and residual silanol groups was dominant, and could afford negative charges for the generation of cathodic EOF. There-

Table 3
Efficiencies and repeatability for four opium alkaloids with CMC modified column.

Alkaloids	Efficiency (plate/m)	Repeatability (% RSD)		
		Efficiency ($n = 6$)	Migration time	
			Run-to-run ($n = 6$)	Day-to-day ($n = 3$)
Morphine	92,000	0.7	0.8	1.4
Thebaine	68,000	1.2	0.9	1.5
Narcotine	11,400	1.7	1.2	1.8
Papaverine	132,000	2.2	1.5	2.5

Conditions: columns: CMC-modified capillary column: 60.0 cm total length (40.0 cm effective length) × 50 μm i.d. capillary; bare fused-silica capillary: 52.0 cm total length (32.0 cm effective length) × 50 μm i.d. capillary; mobile phase: 50 mM phosphate buffer at pH 6.0; applied voltage: −15 kV.

fore, it is a great advantageous property of the modified column that the EOF can be controlled by varying the pH value of buffer solutions.

3.1.4. Reproducibility of the CMC modified column

In order to investigate the reproducibility of the capillary modification procedure, three capillaries were prepared on the same day and three capillaries were modified on different days using identical carboxymethylchitosan solution. Using toluene as EOF marker, the EOF which reflected surface chemistry was measured at pH 3.0. The relative standard deviation (RSD) was 2.8% ($n = 3$) for the capillaries made on the same day and 4.5% ($n = 3$) for capillaries prepared on different days.

Run-to-run and day-to-day reproducibilities of modified column were also tested in terms of EOF at pH 3.0, and the RSD values were 1.6% ($n = 6$) and 2.4% ($n = 6$), respectively. After the column had been saved up for one month by filled with nitrogen, the inter-day RSD values of EOF was still smaller than 2.1% ($n = 6$). And the intra-day RSD values of EOF were less than 4.8% in three months. The results indicate that the prepared column is reliable and promising for long-term applications.

3.1.5. Chemical tolerance against solvents

Chemical stability of CMC-coupled capillary was also surveyed. The EOF was determined after being equilibrated with buffer solution or rinsed with various solvents listed in Table 1 for 30 min. And the stability was evaluated on the basis of the variation ratio of EOF. The chemical stability of three columns by different modified methods was noted in Table 1. As shown in Table 1, the EOF of the CMC-modified column kept constant after rinsing with 0.1 mol/L NaOH, 0.1 mol/L HCl, methanol and acetonitrile respectively, and their corresponding variation ratios were below 2%. It also can be seen that the CMC chemically coated column was much more stable than chitosan-coated [30] and CMC physically adsorbed capillary [31], whose degradation ratio was higher than 12% and 6% respectively after rinsing with 0.1 mol/L NaOH or 0.1 mol/L HCl only 15 min. The good tolerance of CMC-coupled column to acid, alkali and organic solvent assured the relatively wider pH range and organic medium to be used in analysis than that of physically adsorbed coating capillary.

3.2. Separation of basic proteins

Basic proteins are usually positively charged under the separation conditions and all could be inevitably absorbed on the inner wall of a fused-silica capillary through electrostatic and hydrophobic interactions [33]. Under acidic condition, the intrinsic hydrophilicity and the positively charged amino groups on lateral chain of CMC could suppress the absorption of proteins and basic compounds onto the OT column surface. The separation of basic proteins on the CMC modified column was evaluated by CEC, four basic proteins including cytochrome C, lysozyme, ribonuclease A and trypsin were used as model compounds. The isoelectric point (p*I*) values of these proteins were listed in Table 2. Due to the general instability of proteins in presence of organic modifiers promoting their denaturation and recipitation, aqueous mobile phase was adopted when proteins were analyzed. Fig. 5a and b shows the electropherograms of four basic proteins at pH 2.5 and pH 3.0 respectively with anodic EOF. Good separation results of the four proteins were obtained. It indicated the CMC-coated column could hinder the interactions between basic analytes and fused silica. Seen from Fig. 5a and b, a better resolution between trypsin and ribonuclease A was obtained at pH 3.0. In view of peak shape, higher efficiency was obtained at pH 3.0. The elution order was trypsin < ribonuclease A < cytochrome C < lysozyme under anodic EOF, that was the same as the isoelectric point values of the proteins

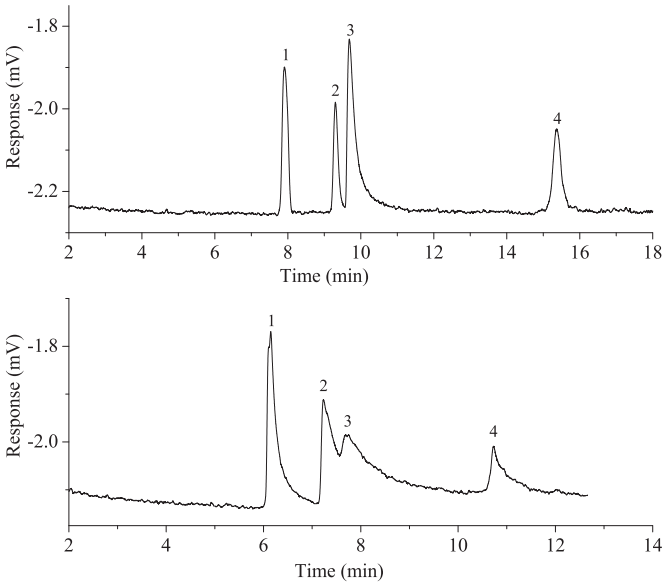

Fig. 6. Separation of opium alkaloids on (a) CMC-modified column and (b) bare fused-silica capillary. Conditions: columns: CMC-modified capillary column: 60.0 cm total length (40.0 cm effective length) × 50 μm i.d. capillary; bare fused-silica capillary: 52.0 cm total length (32.0 cm effective length) × 50 μm i.d. capillary; mobile phase: 50 mM phosphate buffer at pH 6.0; applied voltage: −15 kV; wavelength: 214 nm. Peaks: 1, morphine; 2, thebaine; 3, narcotine; 4, papaverine.

(shown in Table 2). It seemed that protein separation in this CMC coated column was governed mainly by electrophoretic mobility.

The reproducibilities of migration time, peak areas and column efficiency of proteins separation at pH 3.0 of 20 mM phosphate buffer on the CMC modified column were calculated and listed in Table 2. The RSD values for the migration time of four proteins were less than 1.3% for run-to-run ($n = 5$) and less than 3.2% for day-to-day ($n = 3$), and the RSD values for peak areas ranged 3.8–5.6% (run-to-run $n = 5$), 6.8% to 8.8% (day-to-day $n = 3$). It indicated that the separation of proteins using the CMC modified column was reproducible. The column efficiencies of the proteins ranged from 97,000 to 182,000 plates/m. The CMC modified column was proved to be efficient in reducing analyte–wall interactions of basic proteins with the result of good peak shape and high plate numbers.

3.3. Separation of alkaloids

Major opium alkaloids were investigated for the further application of the CMC bonded column. Morphine, thebaine, papaverine and narcotine, which are an important class of pharmacologically active compounds in berbal medicine, could not be easily separated on the uncoated capillary due to the analyte–wall interaction [34]. As shown in Fig. 6b, four alkaloids were not fully resolved with peak tailing in uncoated capillary under the experiment condition. While, Fig. 6a shows the successful separation of four alkaloids on the CMC modified column without obvious peak tailing. Thus, it is obvious that the CMC modified column reduced the analyte–wall interactions. Negatively charged silanol on the fused silica surface were shielded effectively by the CMC modified polymer, moreover, electrostatic repulsion occurred between protonated amino groups on polysaccharide and positive analytes in the acidic condition (pH 6.0). So, the adsorptive action of alkalic analytes onto the silica surface was effectively eliminated. Hereby the CMC modified capillary was suited to separate of opium alkaloids under cathodic EOF. As listed in Table 3, the RSDs of the separation efficiency and migration time for four opium alkaloids were not more than 2.1% and 2.4% respectively, which suggested a satisfactory column repeatability.

4. Conclusion

A capillary modified with hydrophilic polysaccharide carboxymethylchitosan by chemical bonding method was fabricated for open tubular capillary electrochromatography. Compared with conventional physically adsorbed coating, the CMC bonded column exhibited much more remarkable tolerance and chemical stability against 0.1 mol/L HCl, 0.1 mol/L NaOH or some organic solvents. By varying the pH values of running buffer, the CMC modified column could generate both cathodic and anodic EOF. With the prepared column, four basic proteins including cytochrome C, lysozyme, ribonuclease A and trypsin were baseline separated with satisfactory efficiency up to 182,000 plates/m and good reproducibility under anodic EOF. Opium alkaloids were also successfully separated under cathodic EOF without obvious peak tailing. The results showed that the adsorption interactions between basic analytes and the inner surface of capillary could be effectively eliminated on this CMC modified OT column. With some further improvement, CMC-bonded OT capillary column might be an alternative medium for the analysis of basic proteins and alkaline analytes.

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Conflict of interest statement: The authors have declared no conflict of interest.

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