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Capillary electrophoresis separation and permanganate chemiluminescence on-line detection of some alkaloids with β-cyclodextrin as an additive

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Abstract

Capillary electrophoresis has been used in combination with on-line permanganate chemiluminescence detection for the simultaneous determination of morphine, 6-monoacetylmorphine and heroin. It was found that β-cyclodextrins could improve the separation efficiency and enhance the chemiluminescence signal. Improved sensitivity over capillary electrophoresis with UV detection was obtained. The procedure has detection limits of 23, 66 and 115 fmol for morphine, 6-monoacetylmorphine and heroin, respectively.

Keywords: Chemiluminescence detection; Capillary electrophoresis; Alkaloids; Permanganate

1. Introduction

Although capillary electrophoresis (CE) as a separation method has the virtues of high resolution, rapid separation and the ability to analyze small samples, its weakness is thought to be its detection capabilities [1,2]. Most commonly, UV–visible absorption is used as the detection method, with sensitivities typically of 10^{-5}–10^{-6} mol/l. Significantly better limits of detection can be realized when the analytes allow the use of laser-induced fluorescence (LIE) [3,4], amperometric [5] or radiometric [6] detection.

An alternative detection scheme is the use of chemiluminescence, which has been applied to liquid and gas chromatography and to immunoassays [7–12].

Chemiluminescence (CL) should be an ideal spectroscopic detection technique for post-column CE reactions. The excitation energy is generated by a chemical reaction, as opposed to other spectroscopic methods that use an external light source. Therefore, CL should give lower detection limits than other emission procedures, as there is rarely any background emission or scatter to increase the noise level. Because few compounds chemiluminesce there should be minimal interference from endogenous compounds in complex matrices.

However, the poor selectivity of CL greatly limits its application in analysis of complex systems, which could well be overcome by combining CL detection with the CE separation technique. This technique can be directly applied to the determination of trace analytes in complex samples.

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Some twenty applications of capillary electrophoresis–chemiluminescence (CE–CL) have appeared in the literature, particularly, for the determination of proteins [13–15], metal ions [16–18] and amino acids [19], indicating the scope and potential for this hyphenated technique. However, most of the reported CL systems were focused on luminol, peroxyoxalate and an acridinium ester system.

Permanganate, as a strong oxidant in acidic medium, has been commonly used for CL detection. Compared to the most widely used CL reagent, luminol, permanganate has the following advantages: it does not need any catalysts and hydrogen peroxide is also not needed. As it is well known, hydrogen peroxide, one of the most common oxidants that were often combined with luminol, can be easily broken down into bubbles in basic medium. These bubbles can cause an unstable current throughout the electrophoresis process and can even perturb the detection seriously [20]. There will be no such problems existing with the permanganate system. The permanganate CL system also has many advantages over some other commonly used CE combined CL systems including bis(2,4,6-trichlorophenyl) oxalate (TCPO) and acridinium etc. As the most often used CL detection system after CE separation, TCPO generally requires the use of organic solvents because of its poor solubility and CL efficiency in aqueous solution. The toxicity of the organic solutions can be greatly reduced with the permanganate system. For acridinium esters, because they are very susceptible to hydrolysis above pH 3, the pH of the electrophoretic buffer must be strictly controlled.

The main disadvantage of the permanganate system is its relatively poor sensitivity. This is also the main reason that limits its combination with CE. However, this problem could be well solved by choosing suitable enhancement reagents, such as β-cyclodextrins etc. If so, the permanganate CL system will be a relatively ideal detection method for CE.

Cyclodextrins (CDs) are sugar molecules that have the structure of a hollow truncated cone with a hydrophobic cavity. CDs can form a so-called inclusion complex with many species. The high electron density prevailing inside the CD cavity can mobilize the electrons of the included guest molecules, resulting in changes in various spectral properties of both the guest and the CD itself. The effect of CDs on the spectral properties of guest molecules has led to their use as reagents in various spectrometric analyses, including UV–visible spectrophotometric analysis [21], fluorescence and phosphorescence methods [22] and nuclear magnetic resonance spectroscopy [23].

The effect of CDs on the enhancement of CL has been reported. An enhancement of seven-fold for CL of the luminol related compounds was reported by Karatani [24]. Woolf and Grayeski [25] studied the effect of CD solutions on aqueous peroxyoxalate CL. It was found that CDs were capable of increasing the light output by factors up to 300. The enhancement could be attributed to increases in the reaction rate, excitation efficiency and fluorescence efficiency of the emitting species.

Many works for the determination of morphine have been reported. Abbott once established a flow injection CL detection method for morphine [26]. However, this method could only be used for the determination of morphine in very simple systems because of its strong interferences from other related compounds. Later, a HPLC with a CL detection procedure for morphine was developed by Abbott [27]. Though it was very sensitive, investigations were only made for the individual determination of morphine. Yu [28] reported a CE–UV method for morphine and some other opiates, but the sensitivity was much poorer than that developed by Abbott.

Here, we reported a β-CD incorporated CE–permanganate CL detection method for morphine, 6-monoacetylmorphine and heroin. β-CDs were used to improve both the CE separation efficiency and the CL sensitivity for determination of the above mentioned alkaloids. Compared to the methods developed by Abbott, the proposed procedure by us could be used for the simultaneous determination of morphine, 6-monoacetylmorphine and heroin, which have similar structures. Also, improved sensitivity over CE–UV detection was obtained.

2. Experimental

2.1. Apparatus

The CE–CL apparatus used here consisted of a conventional CE system and a CL detection system

as described previously in detail [18]. A 0–30 kV power supply (Department of Chemistry, Beijing University, Beijing, China) provided the separation high voltage. A capillary (56 cm×50 μm I.D.) was used for separation. A 10-cm section of one end of the separation capillary was burned and then etched with 0.1 mol/l HF for 2 h. The HF treated end of the separation capillary was then inserted into a reaction capillary (25 cm×320 μm I.D.).

These two capillaries were held in place by a Plexiglass four-way joint. The required CL reagents were delivered by gravity through a reagent capillary (40 cm× 320 μm I.D.). The outlet of the reagent capillary was also led to the four-way joint.

Plexiglass nuts and polyimide ferrules were used to fix the above mentioned three capillaries inside the four-way joint. The grounding electrode was also put into the joint to complete the CE electrical circuit. The outlet of the reaction capillary was 2 cm lower than the other end to make the solution flow out of the reaction capillary more easily and quickly.

A 1-cm detection window was formed on the reaction capillary (starting 3 mm before the point where the inner separation capillary terminated) by burning off the polyimide coating. The CL emission was collected with a Hamamatsu photomultiplier tube (PMT) (Hamamatsu Photonics, Iwata-Gun, Japan) 1 cm in front of the detection window. The PMT was operated at 850 V and the photocurrent was magnified by a signal magnifier (Institute of Chinese Academy of Sciences, Beijing, China) and was then recorded using a 3066 chart recorder (The Fourth Instrumental Factory of Sichuan, Sichuan, China). The whole CL detection system was held in a large light-tight wood box to exclude stray light.

All the capillaries used were purchased from Hebei Optical Fiber Factory (Hebei, China).

A UVIKON Spectrophotometer 941 plus (Zurich, Switzerland) was used to complete all the fluorescence emission spectra scanning.

2.2. Chemicals

All the alkaloids were obtained from the National Drugs Laboratory of China (Institute for Drugs and Biological Products Testing, Beijing, China). Their chemical structures are listed in Fig. 1. All other reagents were of analytical reagent grade, and the water used was double distilled. All the solutions used were filtered through a 0.22-μm pore-size membrane filter before use.

3. Procedures

The new capillaries were rinsed sequentially with 0.1 mol/l NaOH, distilled water, 0.1 mol/l nitric acid and distilled water for 10 min, respectively, and then were equilibrated overnight with the electrolyte. Electrophoretic separation was performed in 25 mmol/l borate buffer (pH 9.3) containing 0.005 mol/l β-CDs. A CL solution of 6.0×10^{-4} mol/l permanganate and 0.005 mol/l β-CDs was prepared in 1.0 mol/l $H_6P_4O_{13}$. The separation capillary, reagent capillary, four-way joint and the reaction capillary were initially filled with the electrophoretic

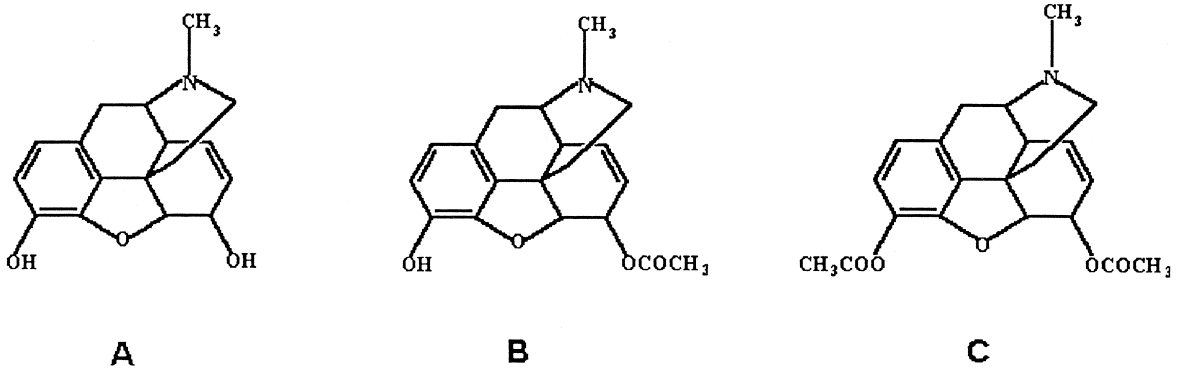

Fig. 1. The chemical structures of the alkaloids. A: morphine; B: 6-monoacetylmorphine; C: heroin.

buffer by means of a syringe. The CL solution was placed 20 cm above the four-way joint during the analysis.

4. Results and discussion

Most of the previously used interfaces for CE–CL detection were post-column [29], which is similar to the originally developed detector by Rose and Jorgenson [30]. In this kind of system, the high voltage electrode was put in the buffer reservoir into which one end of the separation capillary was immersed and the grounding electrode was put behind the reaction capillary.

The bubble problem mentioned in the Section 1 was very serious with this kind of interface [31,32]. However, this problem could be solved to a great extent by putting the grounding electrode before the reaction capillary as shown in Fig. 2. This has been described previously by us in detail [20].

Usually, the mixing of the CL reagents with the analytes out of the separation capillary causes strong perturbation of the flowing electrophoretic stream, which often affects the stability of the electrophoretic current. This problem could also to some extent be reduced with the proposed apparatus, which should be at least partly due to the exclusion of the reaction capillary from the electrophoretic current circuit. For this reason, though no hydrogen peroxide was used this time, we still recommend using this kind of device to complete the CE–CL detection process.

Also, the author has to point out here that the above mentioned perturbation will result in strong diffusion of the analytes out of the separation capillary, which will lead to a broadened band. This should be considered throughout the experiments as a factor causing band broadening.

Morphine, 6-monoacetylmorphine, heroin and some other alkaloids could be oxidized by permanganate in acidic medium and will produce a CL signal. This has been used for the flow injection determination of morphine [26].

Here, we combined this permanganate CL system to the CE separation technique to separate and detect these alkaloids.

The experimental conditions, such as permanganate concentration, acid concentration, migration pH and chemical composition of the migration buffer, were investigated using a sample solution of morphine.

With higher concentrations of permanganate, stronger CL signals could be obtained provided that the permanganate concentration was below 0.6 mmol/l. When the concentration was above this level the CL intensity decreased, at least partly because of the absorption of the emitted light by the colored permanganate. Thus, 0.6 mmol/l was finally selected.

Polyphosphoric acid showed the best medium for

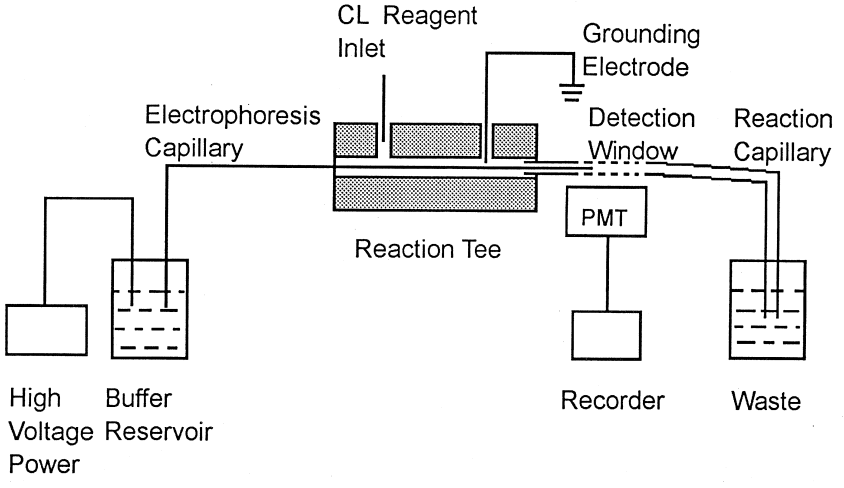

Fig. 2. Schematic diagram of the apparatus for the CE–CL detection.

this CL system among sulfuric acid, hydrogen chloride, nitric acid, phosphoric acid and polyphosphoric acid. With higher concentrations of polyphosphoric acid, stronger CL signals can be obtained, but the viscosity of the solution will increase, which will slow down the flowing of the solution inside the capillaries. For this reason, 1.0 mol/l was finally chosen as a compromise between signal and viscosity.

It is also important to select a proper electrophoretic buffer that is compatible with the detection reaction. Among the electrophoretic buffers tested (borate, phosphate and carbonate), 25 mmol/l borate buffer at pH 9.3 was found to be the optimum, as judged by the stability of the background CL intensity and the detection sensitivity of morphine.

Under the above optimized conditions, three alkaloids (morphine, 6-monoacetylmorphine and heroin) were separated and determined. It can be found from the electropherogram (Fig. 3) that morphine and 6-monoacetylmorphine were not separated efficiently because of their similar molecular structures and that the CL signal was weak. Moreover, a broadened peak can be observed, which may be caused by the peak overlap of morphine and 6-monoacetylmorphine.

Sodium dodecyl sulfate (SDS) was a commonly used surfactant added to the CE separation system to improve the separation efficiency. SDS was also tested by us to see whether it could separate morphine and 6-monoacetylmorphine well. The result

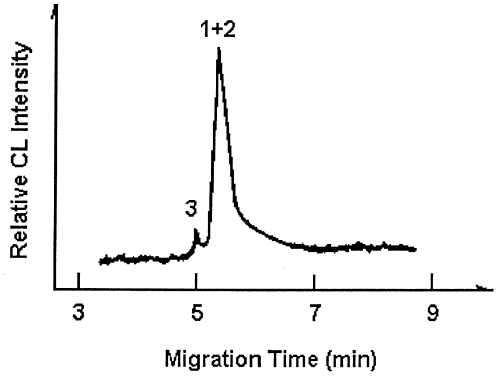

Fig. 3. Electropherogram of three alkaloids. Peak identities: 1= morphine, 2=6-monoacetylmorphine, 3=heroin. Separation voltage: 18 kV; Sample injection, 10 s at 18 kV.

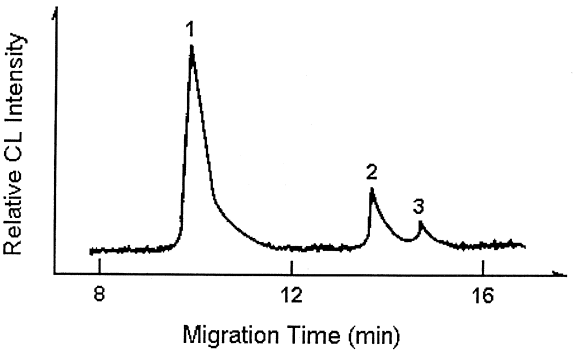

Fig. 4. Electropherogram of three alkaloids with SDS as an additive. SDS concentration: 50 mmol/l; other conditions as for Fig. 1.

(Fig. 4) shows that to some extent SDS improved the separation of morphine and 6-monoacetylmorphine, but the migration time became longer and the peak broadening was still serious.

The present electrophoretic separation was performed under basic conditions (pH 9.3) so that the alkaloids may be neutral. The improvement of the separation efficiency should be attributed to hydrophobic effects. This has been described by Terabe [33].

In untreated fused-silica, the electroosmotic flow, directed towards the cathode, is substantial at pH values ranging from mildly acidic through to alkaline. On the other hand, SDS micelles are anionic and electrophorese towards the anode. As a result, the overall micellar velocity is reduced compared with the bulk flow. This may be the main cause of the longer migration time.

It was reported by Davis [34] that operating at higher SDS concentrations may result in significant Joule heating, which is often one of the major reasons for bandbroadening. The concentration of SDS used in this system is 50 mmol/l, which is much higher than its critical micelle concentration (CMC) (8.1 mmol/l). Maybe this is part of the reason for the peak broadening in Fig. 4.

Longer migration time, which usually to some extent cause solute diffusion, may be another attribution to the peak broadening.

As a conventional surfactant, the so-called CMC must be reached for the surfactant molecules to spontaneously organize into roughly spherical to

ellipsoidal aggregates known as micelles. However, this is not needed for β-CDs. CDs represent another class of additives known to form a pseudophase without needing to form aggregates.

Thus, β-CDs were added to the electrophoretic buffer to see whether it could improve the separation of morphine and 6-monoacetylmorphine. The expected experimental result was obtained. Meanwhile, it was also found that the CL signal could be enhanced. To obtain stronger CL intensity, β-CDs were also added to the post-column CL reagent. The effect of the concentration of β-CDs was also evaluated by changing their concentrations in both the electrophoretic buffer and the post-column CL reagents at the same time. Concentrations above 0.005 mol/l was found to be the optimum, as judged by the separation efficiency and the CL intensity. Therefore, 0.005 mol/l was finally selected. Fig. 5 shows the electropherogram of the above mentioned three alkaloids with the CE–CL system containing β-CDs. It is very clear that both the separation efficiency and the detection sensitivity were greatly improved. These should be due to the hydrophobic cavity of the CDs, which is similar to that of micelles. Solutes may partition into and out of the cavity, and the migration velocities of the solutes can be affected as well. When the solutes partition into the cavities their velocities are retarded. When present in the bulk phase or the interstitial space between β-CDs, the solutes run a normal electrophoresis. The differences of polarity, size and structure of the solute molecules cause the differences in their behavior of partitioning into and out of the β-CDs cavities, which finally result in the differences in the migration velocities of the solutes and in the improvement of the separation efficiency.

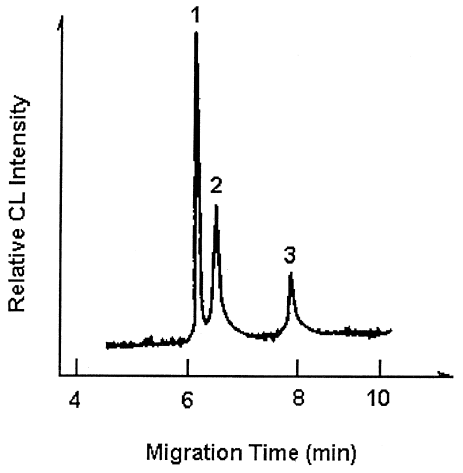

Fig. 5. Electropherogram of three alkaloids with β-CDs as an additive. β-CD concentration: 0.005 mol/l; other conditions as for Fig. 1.

The mechanism for the enhancement of the CL signal by β-CDs was also investigated. CL efficiency is at least partly related to the fluorescence efficiency of the emitting CL reaction products. Oxidation of morphine (e.g., by hexacyanoferrate(III) in alkaline solution [35,36]) produces mainly highly fluorescent dimer pseudomorphine and a small amount of morphine *N*-oxide [37]. This reaction has been used for post-column fluorescence detection of morphine after HPLC separation. We are not certain that the main reaction product for this CL system is the dimer pseudomorphine, but we could conclude from the fluorescence emission spectra of the reaction products of the permanganate–morphine CL system in the polyphosphoric acid medium in the presence and absence of β-CDs (Fig. 6) that the fluorescence intensity of the reaction products is apparently increased in the presence of β-CDs. This partly explains the improvement in the CL efficiency.

Some other reasons for the improvement of the CL efficiency by β-CDs may be increased reaction rate and improved excitation efficiency of the emitting species.

The three alkaloids, morphine, 6-monoacetylmorphine and heroin, were successfully separated under the optimized CE–CL conditions. The following detection limits, defined as three times the standard deviation of the background, were obtained: morphine 23 fmol, 6-monoacetylnorphine 66 fmol and heroin 115 fmol. An improvement of two to five-fold in the sensitivity over CE–UV [28] was obtained.

Methods for the determination of morphine in body fluids are under investigation.

Acknowledgements

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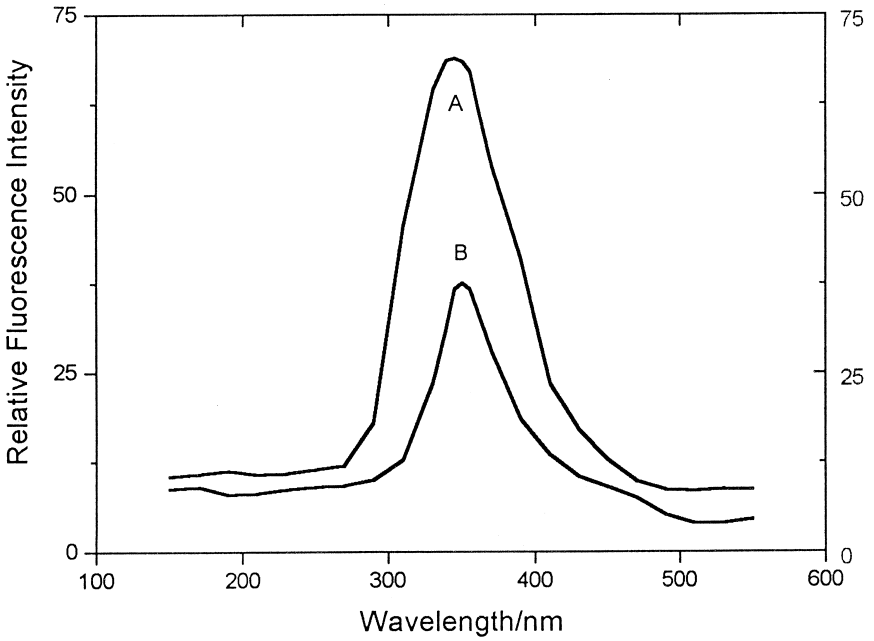

Fig. 6. Fluorescence emission spectra of the mixture of 0.6 mmol/l $KMnO_4$ and 4×10^{-4} mol/l morphine in 1.0 mol/l polyphosphoric acid. Excitation wavelength 290 nm. (A) In the presence of 0.005 mol/l β-CDs. (B) In the absence of β-CDs.

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