Full Paper

Simultaneous Electrochemiluminescence Detection of Anisodamine, Atropine, and Scopolamine in *Flos daturae* by Capillary Electrophoresis Using β-Cyclodextrin as Additive

Jianguo Li,[a, b] *Yuan Chun*,[a] *Huangxian Ju*[a]*

[a] Key Laboratory of Analytical Chemistry for Life Science (Education Ministry of China), Department of Chemistry, Nanjing University, Nanjing 210093, P. R. China
*e-mail: hxju@nju.edu.cn
[b] School of Chemistry and Chemical Engineering, Suzhou University, Suzhou 215006, P. R. China

Received: April 2, 2007
Accepted: May 21, 2007

Abstract

This work developed a simple and sensitive method for simultaneous determination of three effective ingredients, atropine, scopolamine and anisodamine, in *Flos daturae* based on capillary electrophoresis coupled with electrochemiluminescence detection. β-Cyclodextrin was used as an additive to the running buffer for obtaining the absolute separation. The proposed method displayed the linear ranges from 0.2 to 100, 0.2 to 100 and 20 to 200 μM for anisodamine, atropine and scopolamine with correlation coefficients more than 0.99, respectively. This method showed the relative standard deviations less than 4% and 6% for detection of migration time and peak height, respectively, and was suitable for the determination of these tropane alkaloids in plants and valuable in clinical and biochemical laboratories for quality control.

Keywords: Capillary electrophoresis, Electrochemiluminescence, β-Cyclodextrin, Anisodamine, Atropine, Scopolamine

DOI: 10.1002/elan.200703903

1. Introduction

The tropane alkaloids: atropine, scopolamine and anisodamine (shown in Fig. 1) are main effective ingredients in the Chinese herb *Flos daturae* and other solanaceous plants [1, 2]. They have similar structures and are of considerable therapeutic importance as parasympatolytic, anticholinergic and antiemetic drugs. These drugs have been extensively used in clinic, especially in cases of toxic shock and organophosphorus intoxication [3, 4]. Therefore, it is very important to develop a rapid, simple, sensitive and accurate method for the determination of these alkaloids. A review on the current methods for detection of tropane alkaloids has been published [5]. These methods include different separation techniques such as thin layer chromatography (TLC) [6, 7], gas chromatography (GC) [8, 9] and high performance liquid chromatography (HPLC) [10–15]. However, both the TLC and the GC methods show low resolution and poor reproducibility. Although the HPLC for detection of these tropane alkaloids has relatively high efficiency, it needs a large amount of organic solvent as mobile phase, and unknown ingredients in Chinese traditional medicine easily contaminate the chromatographic column. Also, a basic component has to be added into the mobile phase as a masking agent in order to reduce the peak tailing and improve the column efficiency. Furthermore, some complex sample purification steps are frequently needed.

In recent years, capillary electrophoresis (CE) has been widely applied for the analysis of pharmaceutical compounds because of its efficiency, flexibility, accuracy and high resolution. Some reports have been dedicated to tropane alkaloids analysis by capillary electrophoresis [16–27]. A validated capillary zone electrophoresis method coupled with ultraviolet detection has been described for the determinations of atropine and scopolamine in ophthalmic solutions [18] and the main tropane alkaloids in *Hyoscyamus muticus* [24–26], *Datura metel* [27] or *Flos daturae* [28] plants. Hyoscyamine and scopolamine in plant extracts can also be detected using micellar electrokinetic chromatographic method with ultraviolet detection [22, 23]. This work proposed a novel method for simultaneous determination of atropine, scopolamine and anisodamine

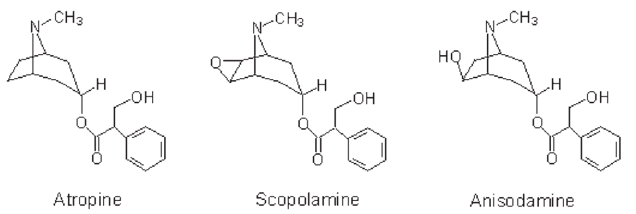

Fig. 1. Structures of atropine, scopolamine and anisodamine.

in *Flos daturae* plants with CE coupled with electrochemiluminescence (ECL) detection using β-cyclodextrin as a buffer additive to improve their separation.

ECL method has been developed for the determination of atropine [29] and anisodamine [30]. Although a CE-ECL method based on $Ru(bpy)_3^{2+}$/tertiary amine system has been established for simultaneous determination of atropine and scopolamine in *Flos daturae* [31], to our best knowledge the simultaneous CE-ECL determination of these three compounds has not been reported yet, the sensitivity, separation efficiency and practicability for tropane alkaloids also need to be further improved. Here three main effective ingredients, atropine, scopolamine and anisodamine, were completely separated by adding β-cyclodextrin to the CE running buffer, which changed the electroosmotic flow (EOF) of analytes inside capillary [20, 21, 32–34], and sensitively detected by using $Ru(bpy)_3^{2+}$ ECL system. This method could be used in rapid and highly selective monitoring anisodamine, atropine and scopolamine in small volume of plant extracts, indicating that it was practical and valuable in clinical and biochemical laboratories for pharmaceuticals analysis and quality control.

2. Experimental

2.1. Materials

All reagents and chemicals used were commercially available and of analytical grade. Scopolamine hydrobromide, anisodamine hydrobromide, atropine sulfate and *Flos daturae* samples were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tris (2,2′-bipyridyl)ruthenium(II) chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). β-Cyclodextrin (recrystallization before use) was obtained from Shanghai Biochemical Co. All solutions were prepared with water purified in a Milli-Q System (Millipore, Bedford, MA). The stock solutions were stored in the refrigerator (4 °C). Standard solutions of atropine, scopolamine and anisodamine were prepared by appropriate dilution of the stock solution with water. All standard solutions and phosphate buffers were weekly prepared and filtered through 0.22 μm cellulose acetate filters (Shanghai Xinya Purification Material Factory) prior to injection.

2.2. Apparatus and Procedures

A programmable high-voltage power supply (0–20 kV, Remax Electronic Co., Ltd., Xi'an, China) was applied to perform the electrokinetic sample injection and electrophoretic separation. An uncoated fused-silica capillary with 50 cm length, 25 μm i.d. and 360 μm o.d. was used for separation (Yongnian Optical Fiber Factory, Hebei, China). Before use, the capillary was flushed with 0.1 M sodium hydroxide solution over night. The electrochemical measurements in CE-ECL experiments were carried out on a MPI-A multifunctional electrochemical analytical system (Xi'an Remex Electronic and Technological Co.) with a three-electrode system comprising platinum wire as counter, Ag/AgCl (3.0 M NaCl) as reference and a 500-μm platinum disk as working electrodes. The ECL emission was detected with a Model BPCL Ultraweak Chemiluminescence Analyzer (Institute of Biophysics, Beijing) in a pulse mode, which was sensitive to photons with a wavelength range of 200 to 800 nm. The working electrode was adjusted and fixed by three screws from three different directions to align with the capillary under the microscope. The gap between working electrode and capillary was controlled at 70 ± 5 μm [35, 36]. The reference and counter electrodes were inserted into the solution above both the capillary and the working electrode. The lower layer of cell was made of a piece of optical glass through which the photons were captured by PMT, which was biased at 800 V. 450 μL 50 mM pH 7.0 phosphate buffer containing 5.0 mM $Ru(bpy)_3^{2+}$ was added to the cell for CE-ECL detection.

Electrophoresis in the capillary was driven by a high-voltage power supply (16 kV, 5 μA), which was applied at the injection end with the detection cell held at ground potential through the separation capillary guide. During the experiment, the separation voltage was applied at the injection end, with the reservoir in the ECL detection cell held at ground potential, and the detection potential was applied at the working electrode. In all experiments, sample introduction was accomplished by electrokinetic injection for 10 s at 10 kV (about 4.5 μL) [35]. Before use, the capillary was flushed with purified water and the running buffer for 15 min by means of a syringe. The running buffer (pH 7.0) contained 20 mM phosphate and 4.0 mM β-cyclodextrin. After each run the electrode was treated with a cyclic voltammetric scan in a potential range of – 0.5 to 0 V at 100 mV/s for 2 min [35, 37], ascertaining to get better resolution and reproducibility. After a stable baseline ECL signal was reached, the sample introduction was performed and the electropherogram was recorded.

2.3. Sample Preparation

The extraction procedure of *Flos daturae* used here was a modification of the technique reported by Kursinszki [15] and Mateus et al. [22]. About 0.5 g powdered herbal medicine was weighted accurately and transferred to 20 mL tube. 8 mL mixture of chloroform-ethanol-concentrated ammonium hydroxide (15:5:1, v/v/v) was added to the tube with sonication for 20 min followed with centrifugation (3000 rpm × 10 min). The extraction step was repeated for three times. The supernatants were collected and evaporated under reduced pressure to dryness. The obtained residue was dissolved with water and the solution was filtered through a 0.22 μm cellulose acetate filter for CE-ECL detection.

3. Results and Discussion

3.1. Effect of Applied Potential on ECL

The applied potential was investigated towards scopolamine, atropine and anisodamine by changing from +1.10 to +1.50 V (vs. Ag/AgCl). When the applied potential was less than +1.10 V, light emission was not observed since Ru(bpy) $_3^{2+}$ was not oxidized on the electrode. With the increasing of applied potential from +1.10 V, the ECL intensities for scopolamine, atropine and anisodamine increased and then reached the maximum value, respectively. Anisodamine and atropine were more sensitive than scopolamine, which could be contributed to the stability of epoxy binding structure in scopolamine. The ECL intensity for both atropine and anisodamine reached the maximum values at +1.25 V, while the applied potential for scopolamine was from +1.25 to +1.30 V. More positive applied potential led to the decrease in their ECL responses. Thus, +1.25 V was selected as the optimum potential for the simultaneous ECL detection of anisodamine, atropine and scopolamine. When the working electrode showed an obvious decrease in ECL signal, a cyclic voltammetric treatment of the electrode with a potential range of −0.5 to 0 V at 100 mV/s for 2 min could regenerate the electrode. With this treatment the electrode kept excellent stability of the ECL signal for two months without polishing or any other pretreatment step.

3.2. Effect of pH of ECL Solution and Running Buffer on ECL

The effects of pH value of both the ECL solution and the running buffer on ECL intensity were investigated from pH 5.0 to 10.0. At the same pH of the running buffer and detection solution, with the increasing pH value of the detection solution the ECL intensity increased and showed the maximum values in the pH ranges of 6.5 to 7.5, 7.0 to 8.0 and 7.0 to 8.5 for anisodamine, atropine and scopolamine, respectively (Fig. 2). When pH value was higher than these ranges, the ECL intensity decreased. Therefore, pH 7.0 was selected for their simultaneous detection.

The pH of running buffer is one of the most important parameters for improving selectivity in CE, especially for closely related compounds. When the buffers with pH from 5.0 to 8.0 in absence of β-CD were used, the peaks of atropine and scopolamine overlapped completely. Upon addition of β-CD in running buffer, the separation efficiency would be greatly improved, and the best result occurred at the pH 7.0. The pH of running buffer influenced not only the net charge of the analytes and the electroosmotic flow inside the capillary, but also the interaction between the hydroxyl groups on the rim of β-CD and substituents near the asymmetric center of the analyte, thus, the analytes showed different migration times at various pHs. In order to obtain high response and good separation, pH 7.0 was selected as the pH value of running buffer for following experiments.

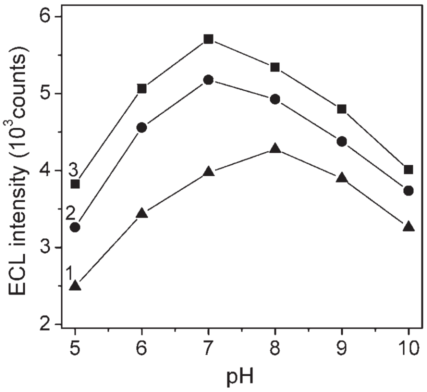

Fig. 2. Effects of pH of ECL cell on ECL responses of 1) 20 μM scopolamine, 2) 5.0 μM atropine, and 3) 5.0 μM anisodamine. Applied potential, +1.25 V; injection, 10 kV for 10 s; running buffer, pH 7.0 20 mM PBS; separation voltage, 16 kV; ECL cell, 5.0 mM Ru(bpy)$_3^{2+}$ + pH 7.0 50 mM PBS; PMT voltage, −800 V.

The concentration of running buffer at 20 mM was found to be the best condition for the separation of these alkaloids in CE–ECL system to obtain low baseline noise.

3.3. Effect of Concentration of β-CD Additive on Resolution

The separation selectivity depends on the relative concentration of β-CD [20, 34]. Figure 3 shows the effect of β-CD concentration on the CE-ECL electropherograms of anisodamine, atropine and scopolamine. The concentration of β-CD in the buffer influenced the selectivity as well as the migration time. With the increasing concentration of additive, the migration time increased and the separation resolution was improved. The separation principle was based on the complexation difference with β-CD among the studied alkaloids. The formation of these complexes reduced the electrophoretic mobility due to their higher mass [20]. The selectivity of atropine and scopolamine was particularly altered. The electropherogram showed the best resolution occurred at the β-CD concentration of 4.0 mM. When the concentration of β-CD was higher than 6.0 mM, the migration times increased to an extent and the EOF of analytes inside capillary changed greatly, which made the peak shape broader [34].

3.4. Effect of Separation Voltage on CE Separation

The increase of separation voltage would increase the current and shorten the analysis time. However, too high separation voltage produced the Joule's heat, which affected the separation of the tested tropane alkaloids. The separation voltage was optimized in the range of 10–18 kV (Fig. 4A). With the increasing separation voltage, the EOF increased and the migration time decreased. The short migration time made more analytes in the effluent arrived in

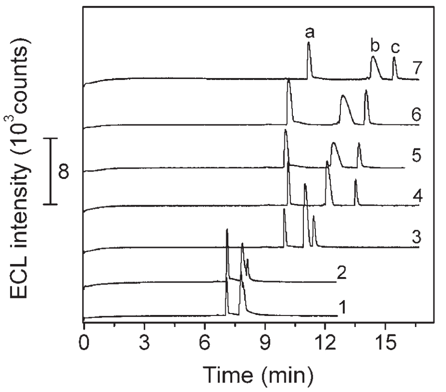

Fig. 3. Effects of β-CD concentration (1: 0 mM, 2: 1 mM, 3: 2 mM, 4: 4 mM, 5: 6 mM, 6: 8 mM, 7: 10 mM) in running buffer on separation of 5.0 μM anisodamine (a), 5.0 μM atropine (b) and 20 μM scopolamine (c) under other optimal conditions.

the diffusion layer of working electrode within a given period [39], thus produced higher ECL signal and better peak shape. This observation was in agreement with the results reported previously [37–40]. Considering the effect of applied voltage on Joule's heat, we selected 16 kV as the separation voltage in this work.

3.5. Limit of Detection, Linearity, and Reproducibility

Using the optimized experimental conditions: applied potential, +1.25 V; separation buffer, 20 mM pH 7.0 PBS containing 4.0 mM β-cyclodextrin; 16 kV separation voltage; sample injection, 10 kV for 10 s; 5 mM Ru(bpy)$_3^{2+}$ in 50 mM pH 7.0 PBS, a typical electropherogram with a good separation of the three standard analytes was shown in Figure 4B. The migration time of three compounds was within 14 min. The theoretical plate numbers (N) for anisodamine, atropine and scopolamine were 57262, 24132 and 56934 plates/m, respectively, calculated with $N = 5.54\,(t_R/W_{1/2})^2$, where t_R is the retention time of the analyte and $W_{1/2}$ is the width of half peak. In the concentration range of 0.2 to 100.0 μM, 0.2 to 100.0 μM and 2.0 to 200.0 μM for anisodamine, atropine and scopolamine, the ECL intensity was proportional to the concentration with the correlation coefficients more than 0.99, respectively. The limits of detection were determined to be 1.0×10^{-8} M (45 amol) for anisodamine, 1.6×10^{-8} M (72 amol) for atropine and 2.0×10^{-7} M (0.9 fmol) for scopolamine at a signal to noise ratio of 3, respectively. Although a compromise for simultaneous determination of three tropane alkaloids was needed, the limits of detection were lower than those of 2.0 μg/mL (4×10^{-6} M) for anisodamine using microchip CE [30], 5.0×10^{-8} M for atropine and 1.0×10^{-6} for scopolamine [31] with CE-ECL system. A standard mixture solution containing 5×10^{-6} M anisodamine, 5×10^{-6} M atropine and 2×10^{-5} M scopolamine was injected consecutively five times to determine the repeatability of ECL intensity based on peak height and migration time for the alkaloids. Relative standard derivations of the ECL peak intensity and the migration time were 2.8% and 1.7% for anisodamine, 5.3% and 3.6% for atropine and 3.4% and 2.5% for scopolamine, respectively. The high reproducibility indicated that this approach was validated for detection of tropane alkaloids.

3.6. Application

The developed method was applied to the analysis of anisodamine, atropine and scopolamine in the extract of *Flos daturae* under the optimized conditions. Identification of the analytes in herbal extracts was confirmed by comparing the electropherogram of diluted extract with that of the extract spiked with anisodamine (a), atropine (b) and scopolamine (c), where the increase of peak height at certain migration time is directly proportional to the amount spiked anisodamine, atropine or scopolamine. The typical electropherograms were shown in Figure 5, displaying a baseline separation for anisodamine, atropine and scopolamine, which was more effective than that with 80% ethanol as extraction solvent [28, 31]. Thus the mixture of chloroform-ethanol-concentrated ammonium hydroxide in

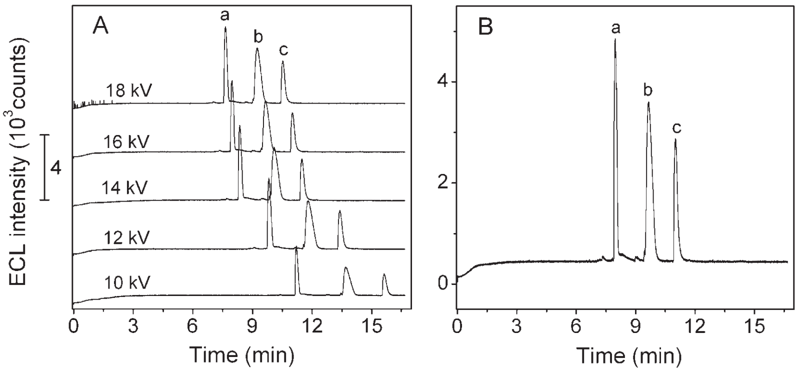

Fig. 4. CE-ECL electropherograms for separation of 5.0 μM anisodamine (a), 5.0 μM atropine (b), and 20 μM scopolamine (c) at different separation voltages (A) and typical electropherogram of 5.0 μM anisodamine (a), 5.0 μM atropine (b) and 20 μM scopolamine (c) under optimal conditions (B).

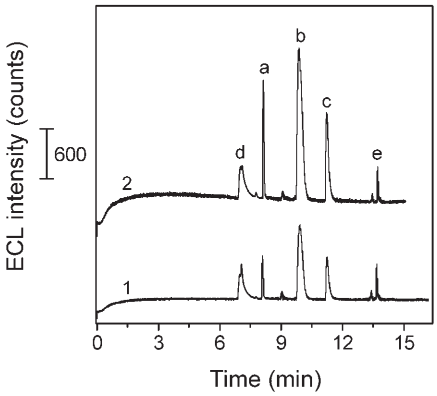

Fig. 5. CE-ECL electropherograms of (1) extract sample of *Flos daturae* and (2) the extract sample spiked with 1.5 μM anisodamine (a), 1.5 μM atropine (b), and 5.0 μM scopolamine (c) under optimal conditions.

15:5:1 (v/v/v) was effective for the extraction of these main effective ingredients in the Chinese herb *Flos daturae*. The other peaks such as peaks d and e observed in the electropherograms of the extract of *Flos daturae* were ascribed to other unknown compounds. Using a standard spike method, the recovery of these compounds was in the range of 83% to 95%, and the contents of the individual constituents in *Flos daturae* were obtained to be 0.82, 1.89 and 4.15 mg/g for anisodamine, atropine and scopolamine, respectively.

4. Conclusions

In this work, a rapid, sensitive and highly selective CE method with simultaneous ECL detection of anisodamine, atropine and scopolamine in the extract of *Flos daturae* is developed. To obtain the good separation for similar structure of these tropane alkaloids, β-cyclodextrin is presently used as a running buffer additive. The optimal conditions are an applied detection potential of +1.25 V, a separation voltage of 16 kV, a sample injection of 10 s at 10 kV, a running buffer of 20 mM pH 7.0 PBS containing 4.0 mM β-CD and a detection solution of 5.0 mM Ru(bpy)$_3^{2+}$ in 50 mM pH 7.0 PBS. Owing to the fast separation, low limits of detection, good selectivity, high sensitivity and powerful resolution, the developed method is suitable and accurate for the detection of tropane alkaloids in plant extract and valuable in clinical and biochemical laboratories for quality control.

5. Acknowledgements

We gratefully acknowledge the financial support of the Distinguished Young Scholar Fund to HX Ju (20325518), Creative Research Groups (20521503) and the Key Program from the National Natural Science Foundation of China (20535010).

6. References

[1] W. C. Evans, in *Trease and Evans' Pharmacognosy* (Ed: W. B. Sanders), London **1996**, pp. 346–358.
[2] M. Lounasmaa, T. Tamminen, in *The Alkaloids*, Vol. 44 (Ed: G. A. Cordell), Academic Press, New York **1993**, pp. 1–114.
[3] Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences, *Natl. Med. J. China* **1973**, *53*, 269.
[4] J. G. Hardman, L. E. Limbird, in *Goodman and Gilman's Pharmacological Basis of Therapeutics*, McGraw Hill, New York **1995**, pp. 141–160.
[5] B. Drager, *J. Chromatogr. A* **2002**, *978*, 1.
[6] R. A. De Zeeuw, J. P. Franke, M. Van Halem, S. Schaapman, E. Logawa, C. J. P. Siregar, *J. Chromatogr. A* **1994**, *664*, 263.
[7] X. P. Luo, X. Yang, S. B. Li, *Chinese Traditional Patent Medicine* **1995**, *17*, 11.
[8] A. Martinsen, T. Naaranlahti, M. L. Turkia, T. Lehtola-Oksanen, J. M. Ylinen, *Phytochem. Anal.* **1991**, *2*, 163.
[9] P. Majlat, *J. Chromatogr. A* **1982**, *241*, 399.
[10] M. Takahashi, M. Nagashima, S. Shigeoka, M. Nishijima, K. Kamata, *J. Chromatogr. A* **1997**, *775*, 137.
[11] O.-W. Lau, C.-S. Mok, *J. Chromatogr. A* **1997**, *766*, 270.
[12] R. Oertel, K. Richter, U. Ebert, W. Kirch, *J. Chromatogr. B* **2001**, *750*, 121.
[13] T. Ceyhan, M. Kartal, M. L. Altun, F. Tülemis, S. Cevheroglu, *J. Pharm. Biomed. Anal.* **2001**, *25*, 399.
[14] H. X. Chen, Y. Chen, P. Du, F. M. Han, H. Wang, H. S. Zhang, *J. Pharm. Biomed. Anal.* **2006**, *40*, 142.
[15] L. Kursinszki, H. Hank, I. László, Éva Szõke, *J. Chromatogr. A* **2005**, *1091*, 32.
[16] I. D. Cruzado, G. Vigh, *J. Chromatogr.* **1992**, *608*, 421.
[17] Y. Y. Rawjee, R. L. Williams, G. Vigh, *J. Chromatogr. A* **1994**, *680*, 599.
[18] S. Cherkaoui, L. Mateus, P. Christen, J. L. Veuthey, *J. Chromatogr. B* **1997**, *696*, 283.
[19] S. Heine, K. Ebert, G. Blaschke, *Electrophoresis* **2003**, *24*, 2687.
[20] S. Cherkaoui, L. Mateus, P. Christen, J. L. Veuthey, *J. Pharm. Biomed. Anal.* **1998**, *17*, 1167.
[21] L. Mateus, S. Cherkaoui, P. Christen, J. L. Veuthey, *J. Chromatogr. A* **2000**, *868*, 285.
[22] L. Mateus, S. Cherkaoui, P. Christen, K. M. Oksman-Caldentey, *Phytochemistry* **2000**, *54*, 517.
[23] S. Cherkaoui, L. Mateus, P. Christen, J. L. Veuthey, *Chromatographia* **1997**, *46*, 351.
[24] L. Mateus, S. Cherkaoui, P. Christen, J. L. Veuthey, *Electrophoresis* **1999**, *20*, 3402.
[25] L. Mateus, S. Cherkaoui, P. Christen, J. L. Veuthey, *J. Pharm. Biomed. Anal.* **1998**, *18*, 815.
[26] M. Eeva, J. P. Salo, K. M. Oksman-Caldentey, *J. Pharm. Biomed. Anal.* **1998**, *16*, 717.
[27] T. Bo, K. A. Li, H. W. Liu, *J. Pharm. Biomed. Anal.* **2003**, *31*, 885.
[28] N. S. Ye, R. H. Zhu, X. X. Gu, H. Zou, *Biomed. Chromatogr.* **2001**, *15*, 509.
[29] Q. J. Song, G. M. Greenway, T. McCreedy, *Analyst* **2001**, *126*, 37.
[30] H. B. Qiu, X. B. Yin, J. L. Yan, X. C. Zhao, X. R. Yang, E. K. Wang, *Electrophoresis* **2005**, *26*, 687.
[31] Y. Gao, Y. L. Tian, E. K. Wang, *Anal. Chim. Acta* **2005**, *545*, 137.
[32] X. H. Xu, J. T. Stewart, *J. Pharm. Biomed. Anal.* **2000**, *23*, 735.
[33] S. Terabe, K. Otsuka, H. Nishi, *J. Chomatogr. A* **1994**, *666*, 295.

[34] I. S. Lurie, *J. Chromatogr. A* **1997**, *792*, 297.
[35] J. F. Liu, J. L. Yan, X. R. Yang, E. K. Wang, *Anal. Chem.* **2003**, *75*, 3637.
[36] W. D. Cao, J. F. Liu, X. R. Yang, E. K. Wang, *Electrophoresis* **2002**, *23*, 3683.
[37] W. D. Cao, J. F. Liu, H. B. Qiu, X. R. Yang, E. K. Wang, *Electroanalysis* **2002**, *23*, 1571.
[38] M. T. Chiang, M. C. Lu, C. W. Whang, *Electrophoresis* **2003**, *24*, 3033.
[39] J. F. Liu, W. D. Cao, X. R. Yang, E. K. Wang, *Talanta* **2003**, *59*, 453.
[40] W. Y. Lee, T. A. Nieman, *Anal. Chem.* **1995**, *67*, 1789.