Journal of Chromatography A, 1217 (2010) 4753–4756

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Determination of the number of binding sites and binding constant between diltiazem hydrochloride and human serum albumin by ultrasonic microdialysis coupled with online capillary electrophoresis electrochemiluminescence

Biyang Deng*, Hua Lu, Linqiu Li, Aihong Shi, Yanhui Kang, Quanxiu Xu

Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources (Ministry of Education of China), College of Chemistry and Chemical Engineering, Guangxi Normal University, Yucai Road 15, Guilin 541004, China

ARTICLE INFO

Article history:
Received 10 February 2010
Received in revised form 4 May 2010
Accepted 10 May 2010
Available online 20 May 2010

Keywords:
Ultrasonic microdialysis
Diltiazem hydrochloride
Capillary electrophoresis
Electrochemiluminescence
Human serum albumin

ABSTRACT

A simple, sensitive and selective determination of diltiazem hydrochloride (DLT) is described using capillary electrophoresis electrochemiluminescence (CE-ECL). The CE-ECL parameters that affect separation and detection were optimized. Under the optimized conditions, the linear range of DLT was from 0.02 to 100 μmol/L (r^2 = 0.9983), with the detection limit of 5.1 nmol/L (3σ). The relative standard deviations of ECL intensity and the migration time were <2% for 0.1 μmol/L and 22 μmol/L DLT (n = 11). A new technique for determining of the number of binding sites and binding constant between DLT and HSA was developed using ultrasonic microdialysis coupled with CE-ECL. The number of binding sites and binding constant were 5.9 and 6.3 × 10^4 L/mol, respectively. The time required for ultrasonic microdialysis was 10 times less than that for traditional dialysis. Compared with traditional dialysis, ultrasonic microdialysis is simple, rapid, and should be applicable to a wide range of interactions of drugs and biomacromolecules.

1. Introduction

Diltiazem hydrochloride (DLT) is widely used as a calcium-channel inhibitor of many cardiovascular diseases, especially coronary heart disease and hypertension [1]. DLT has been determined by liquid chromatography [2,3], capillary electrophoresis [4], NIRS [5], and spectrophotometry [6] methods.

Human Serum albumin (HSA), which is the most abundant protein in the blood circulatory system, plays a very important role in the transport and deposition of a variety of endogenous and exogenous substances, such as fatty acids, hormones, and drugs [7]. HSA binding of drugs has great significance in pharmacology and pharmacodynamics and can affect the biological activity [8] and toxicity [9] of drugs. Knowledge of the binding parameters is helpful for better understanding of the absorption and distribution of drug molecules.

Analytical techniques for studying drug–protein interactions have been developed, such as traditional equilibrium dialysis [10], ultrafiltration [11], high-performance liquid chromatography (HPLC) [12] and high-performance affinity chromatography (HPAC) [13]. All these methods have made contributions to the evaluation of drug–protein interactions. However, a number of shortcomings have emerged in the above techniques, such as long analysis times, large sample size requirements of traditional equilibrium dialysis, long dialysis times and the high operational costs of HPLC and HPAC [14]. Capillary electrophoresis (CE), with the advantages of small sample size, high resolution, short analysis times and low operational costs, has become an important and powerful analytical tool [15]. Electrochemiluminescence (ECL) detection, which combines an excellent detection limit and low cost, is a very suitable technique for CE [16]. CE with ECL detection using $Ru(bpy)_3^{2+}$ has been studied for the determination of a variety of analytes [17–24]. However, samples of biological origin usually contain inorganic constituents and proteins at very high concentrations that can cause problems for CE [25]. Meanwhile, because of its small sample size, high selectivity, real-time monitoring [26] and the continuous nature of the sampling mechanism [27], microdialysis, coupled with HPLC, CE and other analytic methods, has been widely used in biology and pharmacy [28–30].

The interaction of drugs with HSA has been reported using CE-ECL in recent literature [16] but the experimental work has suffered from long dialysis times due to the use of traditional equilibrium dialysis. The ultrasonic technique was used in food industry [31], environment [32] and biology [33]. Ultrasound, at low intensity levels, enhances the movement of the liquid medium

* Corresponding author. Tel.: +86 773 5845726; fax: +86 773 2120958.
E-mail address: dengby16@163.com (B. Deng).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.05.021

and enhances mass transfer because the vibrating gas bubble in an acoustic field generates a circulatory liquid motion around itself, referred to as microstreaming [34]. This enhances the transport of small molecules through the dialysis membrane, increasing the turnover number and thus increasing the rate of approach to equilibrium.

In this work, it was found that the weak ECL signal from the electrochemical oxidization of $Ru(bpy)_3^{2+}$ was greatly enhanced by diltiazem hydrochloride. Based on this observation, CE with ECL detection was applied to the determination of diltiazem hydrochloride. A new method that coupled online ultrasonic microdialysis with CE-ECL was applied to the study of the interaction of DLT and HSA. The number of binding sites and the binding constant were obtained.

2. Experimental

2.1. Reagents and apparatus

DLT was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HAS (>96%, MW 65.3 kDa) and the dialysis membrane (15 kDa) were obtained from Sigma–Aldrich (St. Louis, MO, USA) and Union Carbide Corporation (Chicago, IL, USA), respectively. All solutions were prepared with double-distilled water (DDW) and stored in the refrigerator at 4 °C. Prior to CE analysis, the required sample solutions and phosphate buffer were filtered through 0.45 μm membrane filters.

TU-1901 UV–Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd, Beijing, China.) was used in experiments. All electrophoretic experiments were performed with a 50 cm uncoated silica capillary (75 μm i.d., 375 μm o.d.). All other instruments and chemicals were shown in the literature [23].

2.2. Procedures

350 μL of 5 mM $Ru(bpy)_3^{2+}$ in phosphate buffer (50 mM, pH 7.5) was placed in the ECL detection cell for ECL measurement. The $Ru(bpy)_3^{2+}$-phosphate solution was replaced every 3 h during the experiments to avoid errors in ECL measurement due to possible changes in $Ru(bpy)_3^{2+}$ concentration. The capillary was filled with 1 mol L^{-1} NaOH for 30 min before use and then flushed with filtered water for 30 min using a syringe. Before each run, the capillary was flushed with filtered water and the corresponding running buffer for about 5 min, successively [17].

In all experiments, samples were introduced into the capillary by electrokinetic injection at 12 kV for 8 s, separated in the capillary at 10 kV. Detection potential was fixed at 1.25 V. The running buffer (pH 9.5) contained 10 mmol/L phosphate. The potential of the photomultiplier tube (PMT) was operated at 800 V. After a stable baseline ECL signal was reached, electromigration injection was used for sample introduction and the electropherogram was recorded.

2.3. Ultrasonic microdialysis device

The device was composed of an ultrasonic cleaning instrument, a custom made dialysis bag (approximately 15 μL volume), a plastic tube (inner diameter 1 mm, total length 1.5 cm), a glass dialysis tube (inner diameter 5 mm, about 150 μL volume), a foam, a thermometer and a string (Fig. 1).

The custom made dialysis bag, which was connected with a plastic tube and filled with the DLT-HSA mixed solution, was fixed onto the glass tube by string. The dialysis bag was entirely immersed in dialysis liquid and the dialysis device was installed in the ultrasonic cleaner via the foam. A thermometer was inserted into water for controlling the temperature (<5 °C). The dialysis liquid was determined by CE-ECL.

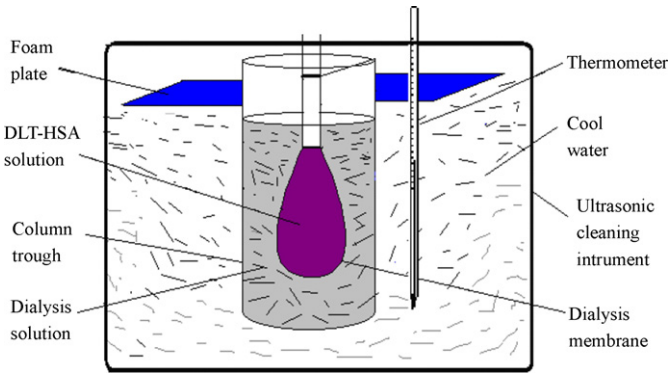

Fig. 1. Equipment of the ultrasonic microdialysis system.

3. Results and discussion

3.1. Cyclic voltammetric scan and detection potential

When there was only $Ru(bpy)_3^{2+}$ in ECL detection, a low ECL intensity was detected. A higher ECL intensity was detected after adding DLT into the ECL detection cell. Therefore, based on this result, a new sensitive method for determination of DLT has been established.

The ECL intensities at a series of voltages were measured. There was a peak intensity when the detection voltage was at 1.25 V; hence, the detection potential was fixed at 1.25 V in subsequent experiments.

3.2. Optimization of pH

The phosphate buffer pH has a very important effect not only in the ECL cell but also in the separation buffer. To obtain the optimal sensitivity and suitable EOF, a series of pH values in the ECL cell and the separation buffer pH were studied, respectively [35]. A phosphate buffer of pH 7.5 and 9.5 were used in the ECL cell and the separation buffer, respectively, throughout the experiments (Fig. 2).

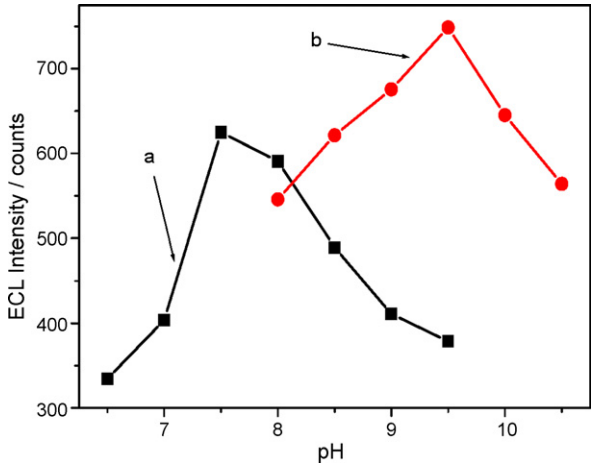

Fig. 2. The effect of the phosphate buffer pH in the ECL cell (a) and the pH of the separation buffer (b) on ECL intensity. Detection conditions in Fig. 5a: 22 μmol/L DLT; electrokinetic injection: 10 kV × 10 s; separation buffer: 12 mmol/L phosphate buffer (pH 8.5); separation voltage: 12 kV. ECL cell contents: 5 mmol/L $Ru(bpy)_3^{2+}$, 50 mmol/L phosphate buffer; detection potential: 1.25 V. Detection conditions in Fig. 5b: pH 7.5 in ECL cell; other conditions are shown in Fig. 5a.

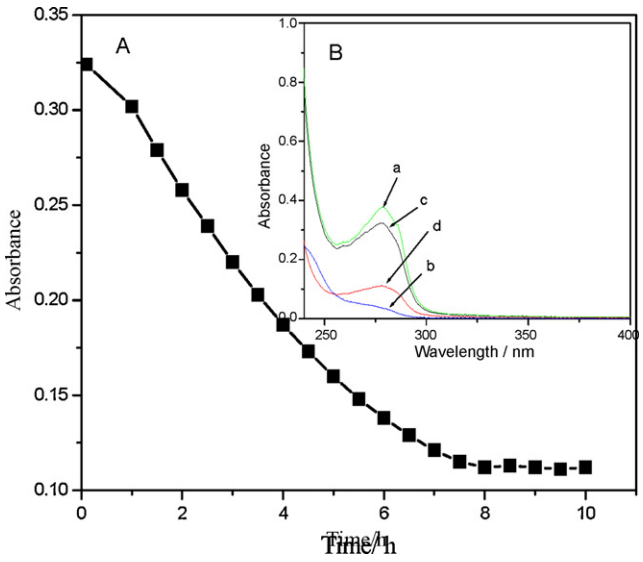

Fig. 3. Effect of incubation time between DLT and HSA on absorbance (A) and UV–Vis spectra (B) of 0.1 mmol/L HSA (a), 0.1 mmol/L DLT (b). Inset: the mixture of HSA and DLT at 0.1 h (c) and 8 h (d).

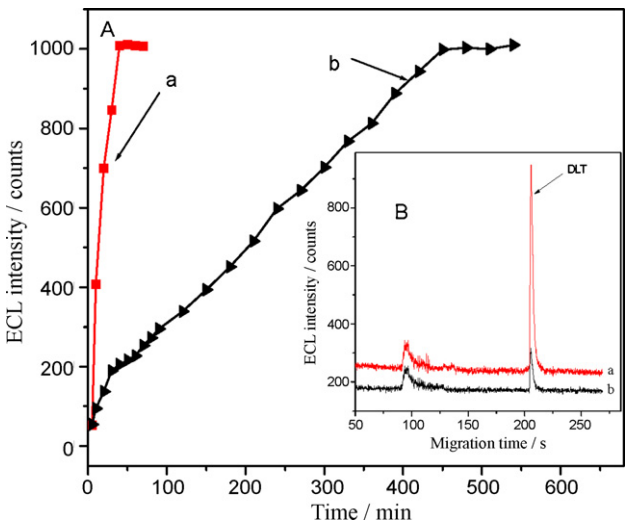

Fig. 4. Effect of dialysis time on ECL intensity (A) and CE-ECL electropherograms (B) for ultrasonic microdialysis (a) and traditional dialysis (b). Detection conditions for Fig. 4A were described in Section 2.2. Detection conditions in Fig. 4B were as described for Fig. 4A. Dialysis time 20 min.

3.3. Effects of injection voltage and separation voltage

The influences of injection voltages of 4, 6, 8, 10, 12, 14 and 16 kV on the ECL intensity were carried out. An optimal injection voltage of 12 kV was recommended as a result.

The ECL intensity increased with separation voltage up to 10 kV, then dropped as the voltage was further increased. Thus, we chose 10 kV as the separation voltage in this experiment to ensure a high ECL intensity as well as good resolution.

3.4. Linearity, detection limit and reproducibility

Under the optimized conditions (seen as Section 2.2), the calibration curve for DLT was linear with concentration in the range from 0.02 to 100 μmol/L, with a regression equation of $y = 33.8 + 59.4x$ ($r^2 = 0.9983$, ×unit for μmol/L). The detection limit was 5.1 nmol/L (3σ). When six consecutive injections of 0.1 and 22 μmol/L DLT were performed, the relative standard deviations (RSD) of the peak height and the migration time were 1.8%, 1.3% and 1.7%, 1.1%, respectively.

3.5. Study on incubation time of HSA with DLT

In order to obtain the optimal incubation time, a mixture solution of 0.1 mmol/L DLT and 0.1 mmol/L HSA was detected by UV–Vis spectrophotometry at room temperature (25 °C). The absorbance decreased with binding time and reached a stable value at 8 h (Fig. 3A). Thus, the incubation time was 8 h. The UV–Vis spectra of the mixture solution are shown in Fig. 3B. When the wavelength was 278 nm, there was no absorbance of DLT. The absorbance decreased as DLT was added into the HSA solution. Both facts confirmed binding between HSA and DLT under the conditions used.

3.6. Comparison of ultrasonic microdialysis and traditional dialysis

Controlled experiments of traditional dialysis and ultrasonic microdialysis were designed, with samples of 10 μL of 0.05 mmol/L DLT solution and 0.01 mmol/L HSA and a dialysis liquid of 100 μL of 10 mmol/L phosphate buffer, pH 9.5. The stirring rate for traditional dialysis was 100 rpm. The frequency and power of the ultrasonic cleaner were 59 kHz and 1 W/cm^2, respectively. The dialysis liquid was determined by online coupled CE with ECL every 10 min (Fig. 4A).

Compared with traditional dialysis, ultrasonic microdialysis could achieve the equilibrium more rapidly by enhancing the mass transfer efficiency. The same ECL intensity measured by the two dialysis methods at the dialysis equilibrium indicates that ultrasonic microdialysis had no influence on the interaction of DLT and HSA. The time required for equilibrium by ultrasonic microdialysis was 40 min, which was far less than that by traditional dialysis (480 min) (Fig. 4A). Ultrasonic microdialysis notably improved the dialysis efficiency by about 10 times. The CE-ECL electropherograms using ultrasonic microdialysis and traditional dialysis are shown in Fig. 4B.

3.7. Study on binding of HSA with DLT using ultrasonic microdialysis

In most cases, the drug is bound to m types of independent binding sites on the protein. The fraction, r, of bound drug molecules per protein molecule is given by Busch as follows [36]:

$$r = \frac{[D_{\text{bound}}]}{[P_{\text{total}}]} = \sum_{i=1}^{m} n_i \frac{K_i[D_{\text{free}}]}{1 + K_i[D_{\text{free}}]} \quad (1)$$

where $[D_{\text{bound}}]$, $[P_{\text{total}}]$ and $[D_{\text{free}}]$ are the concentrations of bound drug, total protein and free drug, respectively; n_i represents the number of sites of class i and K_i is the binding constant. Drug protein data analysis often assumes one type of binding sites on the protein and Eq. (1) can therefore be simplified to:

$$r = n \frac{K[D_{\text{free}}]}{1 + K[D_{\text{free}}]} \quad (2)$$

where n and K correspond to the number of binding sites and the binding constant, respectively [37].

A series of different concentrations of DLT were mixed with 0.01 mmol/L HSA in the custom microdialysis bag and a range of $[D_{\text{free}}]$ were determined after equilibrium of the ultrasonic microdialysis. Therefore, the binding curve was established to estimate the number of binding sites and binding constant (Fig. 5). According to formula (2) and a non-linear fit using Ori-

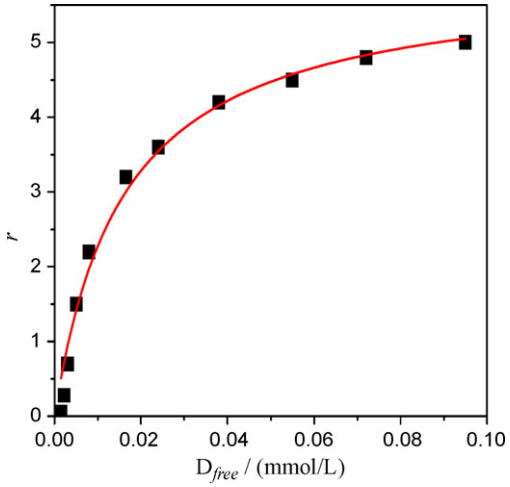

Fig. 5. Binding curve for DLT with HSA.

gin 7.5, the number of binding sites and binding constant can be obtained.

Fitting non-linear equation: $r = 5.9 \times \frac{6.3\times10^4[D_{\text{free}}]}{1+6.3\times10^4[D_{\text{free}}]}$

As shown in the fitting equation, the number of binding sites and binding constant were 5.9 and 6.3×10^4 L/mol, respectively.

4. Conclusions

This paper described a simple, rapid, economical and sensitive CE-ECL method for determination of DLT. A linear DLT response range from 0.02 to 100 μmol/L was obtained, with a detection limit of 5.1 nmol/L (3σ). The determination of the number of binding sites and binding constant between DLT and HSA was developed using a custom-built ultrasonic microdialysis system coupled with CE-ECL. The time required for ultrasonic microdialysis was 10 times less than that for traditional dialysis. Compared with traditional dialysis, the ultrasonic microdialysis is simple, rapid and should be applicable to a wide range of interactions between drugs and biomacromolecules such as proteins and DNA.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (No. 20965001) and Guangxi Science Foundation of China (No. 2010GXNSFA013051, No. 2010GXNSFF013001).

References

[1] J. Li, X. Wang, C. Wang, Q. Fu, L. Liu, M. Huang, S. Zhou, J. Chromatogr. B 867 (2008) 111.
[2] M. Gil-Agustí, S. Carda-Broch, M.E. Capella-Peiró, J. Esteve-Romero, J. Pharm. Biomed. Anal. 41 (2006) 1235.
[3] B. Dasandi, S. Shah, Shivprakash, J. Chromatogr. B 877 (2009) 791.
[4] B. Chankvetadze, I. Kartozia, G. Blaschke, J. Pharm. Biomed. Anal. 27 (2002) 161.
[5] C. Bodson, E. Rozet, E. Ziemons, B. Evrard, P. Hubert, L. Delattre, J. Pharm. Biomed. Anal. 45 (2007) 356.
[6] N. Rahman, S.N.H. Azmi, Microchem. J. 65 (2000) 39.
[7] G. Zhang, Q. Que, J. Pan, J. Guo, J. Mol. Struct. 881 (2008) 132.
[8] N. Seedher, Ind. J. Pharm. Sci. 62 (2000) 16.
[9] D. Silva, C.M. Cortez, J. Cunha-Bastos, S.R.W. Louro, Toxicol. Lett. 147 (2004) 53.
[10] A. Viani, M. Cappiello, D. Silvestri, G.M. Pacifici, Dev. Pharmacol. Ther. 16 (1991) 33.
[11] F. Andreasen, P. Jakobsen, Acta Pharmacol. Toxicol. 35 (1974) 49.
[12] J.Z. Chen, C. Ohnmacht, D.S. Hage, J. Chromatogr. B 809 (2004) 137.
[13] H.S. Kim, I.W. Wainer, J. Chromatogr. B 870 (2008) 22.
[14] N. Zhou, Y. Liang, P. Wang, J. Mol. Struct. 872 (2008) 190.
[15] J. Sun, X. Xu, C. Wang, T. You, Electrophoresis 29 (2008) 3999.
[16] Y. Huang, W. Pan, M. Guo, S. Yao, J. Chromatogr. A 1154 (2007) 373.
[17] B. Deng, A. Shi, L. Li, Y. Kang, J. Pharm. Biomed. Anal. 48 (2008) 1249.
[18] Y. Chen, Z. Lin, J. Chen, J. Sun, L. Zhang, G. Chen, J. Chromatogr. A 1172 (2007) 84.
[19] B. Deng, A. Shi, L. Li, F. Xie, H. Lu, Q. Xu, Microchim. Acta 165 (2009) 279.
[20] B. Deng, L. Li, A. Shi, Y. Kang, J. Chromatogr. B 877 (2009) 2585.
[21] Y. Gao, Y. Xu, B. Han, J. Li, Q. Xiang, Talanta 80 (2009) 448.
[22] H. Li, L. Shi, X. Liu, W. Niu, G. Xu, Electrophoresis 30 (2009) 3926.
[23] Y. Liu, J. Cao, W. Tian, Y. Zheng, Electrophoresis 29 (2008) 3207.
[24] X. Sun, Y. Niu, S. Bi, S. Zhang, Electrophoresis 29 (2008) 2918.
[25] M. Dankova, D. Kaniansky, S. Fanali, F. Ivanyi, J. Chromatogr. A 838 (1999) 31.
[26] J.R. Jimenez, M.D.L. Castro, Trend Anal. Chem. 25 (2006) 563.
[27] L.C. Mecker, R.S. Martin, J. Assoc. Lab. Aut 12 (2007) 296.
[28] R.K. Verbeeck, Adv. Drug Deliv. Rev. 45 (2000) 217.
[29] Y.L. Chang, M.H. Chou, M.F. Lin, T.H. Tsai, J. Chromatogr. A 914 (2001) 77.
[30] S.D. Arnett, D.M. Osbourn, K.D. Moore, S.S. Vandaveer, C.E. Lunte, J. Chromatogr. B 827 (2005) 16.
[31] T.J. Mason, L. Paniwnyk, J.P. Lorimer, Ultrason. Sonochem. 3 (1996) 253.
[32] H. Li, Y. Jin, B.M. Rasool, J. Hazard. Mater. 161 (2009) 1421.
[33] P. Zhang, G. Zhang, W. Wang, Bioresour. Technol. 98 (2007) 207.
[34] J.V. Sinisterra, Ultrasonics 30 (1992) 180.
[35] J. Wang, Z. Peng, J. Yang, X. Wang, N. Yang, Talanta 75 (2008) 817.
[36] M.H.A. Busch, L.B. Carels, H.F.M. Boelens, J.C. Kraak, H. Poppe, J. Chromatogr. A 777 (1997) 311.
[37] A.V. Rudnev, S.S. Aleksenko, O. Semenova, C.G. Hartinger, A.R. Timerbaev, B.K. Keppler, J. Sep. Sci. 28 (2005) 121.