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Protein binding study of clozapine by capillary electrophoresis in the frontal analysis mode

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Abstract

We have used capillary electrophoresis in the frontal analysis mode (CE-FA) to determine the unbound concentration of clozapine in human serum albumin (HSA), human plasma, rabbit serum and plasma sample. The unbound clozapine concentration was directly measured from the height of the frontal peak. Samples were injected directly into an uncoated fused silica capillary (0.65 m (L_C) × 75 μm i.d.; L_E = 0.35 m) and separation was accomplished within 11 min without extensive sample pretreatment. The most suitable running buffer to separate unbound clozapine peak from the other peaks due to endogenous substances was found to contain 1 mmol l^{-1} EDTA, 0.5 mol l^{-1} glycine, and 67 mmol l^{-1} phosphate with pH 7.4. The concentrations of unbound clozapine agreed well with those determined by the conventional ultrafiltration method. The methodology is validated and good correlation and precision are obtained. It was found that clozapine is strongly bound to protein in human plasma, rabbit plasma, and serum, while hardly bound to HSA. The present method enables the determination of the unbound drug concentration in multiple equilibrium system with less than ultra-micro injection volume, and would be hence especially useful for the protein binding study in biological samples that are only available in minute quantities.

Keywords: Human serum albumin; Human plasma; HPCE-FA (high performance capillary electrophoresis-frontal analysis); Rabbit plasma and serum

1. Introduction

The overall protein binding of a drug involves multiple equilibria, which all together affect the unbound (free) drug concentration in blood and thus, the distribution, metabolism, elimination, and pharmacological effect of drugs. There are numerous methods applicable for study on drug binding to plasma proteins [1–5]. Current high performance capillary electrophoresis (CE) is one of the most dynamically growing analytical techniques in studying molecular interactions because of its speed, efficiency, and selectivity. CE offers several modes for the quantitative assessment of protein–drug interactions depending on the stability of the complex or the on-and-off kinetics of binding reaction [6]. If the mobility of the protein is equal to the mobility of the complex, capillary electrophoresis-frontal analysis (CE-FA) is the

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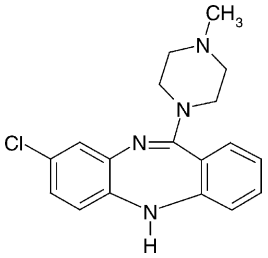

Fig. 1. Chemical structure of clozapine.

most favorable method among them to investigate molecular interaction [7–10].

The CE-FA method is simple, robust, easy to implement, can deal with multiple equilibria and requires less material (nl). In addition, the results of the CE-FA method agree very well with those obtained with conventional methods [7]. Based on this, The CE-FA method has successfully been applied to investigate the interaction of drugs with HSA [11], plasma lipoproteins [12], α_1-acid lipoprotein (AGP) [13], protein in human plasma [14], etc. The principle and main features of CE-FA method were described in the literature 7.

Clozapine (Fig. 1), a tricyclic neuroleptic and antidepressant, is highly protein-bound in serum [15]. It has proven to be very effective in the treatment of therapy resistant psychotic patients and patients who are suffering from the extra pyramidal side (EPS) effects of the classic antipsychotics. It was reported that the binding affinity of clozapine to AGP is stronger by two orders of magnitude than that to HSA [16]; the overall human plasma protein binding property of clozapine has been investigated by equilibrium dialysis followed by HPLC [15].

The present study investigated the separation of unbound clozapine from HSA, human plasma proteins, rabbit plasma proteins and serum proteins by CE-FA method. The factors affecting the electrophoretic separation and the applied range of present method were discussed.

2. Experimental

2.1. Chemicals

Human serum albumin (HSA, fatty acid free) was purchased from Sigma (St. Louis, MO, USA). Clozapine with purity >99.5% was supplied by the Institute of Pharmaceutical and Biological Product Control (Beijing, China). All other chemicals were of analytical grade and purchased from Shenyang Chemicals (Shenyang, China). The water was always double distilled. Phosphate buffer (67 mmol l^{-1}, $I = 0.17$ mol l^{-1}) of physiological pH (7.4) was used as the sample solvent. Uncoated fused silica capillary (75 μm i.d.) was purchased from Ruifeng Chromatographic Product Co. (Yongnian, Hebei, China).

2.2. Equipment

All CE experiments were performed using an HV-301 HVPS Capillary Electrophoresis System (Younite Unimicro Technologies, Co. Ltd, Tongliao, Inner Mongolia, China) with a VUV-22 UV detector fixed at 214 nm. The power supply was operated in voltage-controlled mode. The operating parameters of CE are given in Table 1. The electrophoretic data were acquired with a Jiangshen workstation (Dalian, China).

2.3. Preparation of rabbit serum and plasma

The blood was drawn in tubes containing potassium oxalate and sodium fluoride as anticoagulant and immediately centrifuged, and the plasma was separated; serum was obtained in tubes with no anticoagulant, the blood was allowed to clot at room temperature for 20 min and then centrifuged. The plasma and serum were divided into aliquots and stored frozen until used in the binding experiments.

Table 1
Operating parameters of CE

Parameter	Setting
Fused silica capillary	0.65 m (L_C) × 75 μm i.d; L_E = 0.35 m
Analyte	200 μmol l^{-1} clozapine in plasma or serum
Analyte injection method	Hydrostatic injection; Δh = 0.11 m, 30 s
Detection	UV at 214 nm
Voltage applied	6 kV
Temperature	Room temperature
BGE	Potassium phosphate buffer (pH 7.4, 67 mmol l^{-1} containing 0.5 mol l^{-1} glycine and 1 mmol l^{-1} EDTA)

2.4. Human serum

Pooled plasma from healthy donors was collected on citrate–phosphorus–dextrose (CPD) (PVC bags from Medical Macromolecular Manufacture, Tianjin, China).

2.5. Preparation of sample solutions

The running phosphate buffer was prepared by mixing 10 parts of 67 mmol l^{-1} dipotassium hydrogen phosphate and one part of 67 mmol l^{-1} potassium dihydrogen phosphate, EDTA and glycine were added to the mixture at a concentration of 1 mmol l^{-1} and 0.5 mol l^{-1}, respectively; isotonic phosphate buffer was prepared by mixing 10 parts of 67 mmol l^{-1} dipotassium hydrogen phosphate and one part of 67 mmol l^{-1} potassium dihydrogen phosphate with the isotonicity of each solution being adjusted with approximately 5 and 4 mg/ml of NaCl, respectively, yielding an ionic strength of 0.17 mol l^{-1} and pH 7.4. The obtained running phosphate buffer and isotonic phosphate buffer were degassed by sonication in ultrasonic water bath (DL_180, Shipuhaitian Electronical Instrument Co., Xiangshan, Zhejiang, China) for 25 min and filtered through a 0.45 μm membrane filter before use. HSA solution of (550 μmol l^{-1}) in 67 mmol l^{-1} isotonic phosphate buffer (pH 7.4; I = 0.17 mol l^{-1}) was prepared by adding the appropriate volume of buffer to a weighed amount of HSA. Stock solution for clozapine was prepared by dissolving the reference standard in methanol. Appropriate volumes of the stock solution were pipetted into tubes, evaporated to dryness under a stream of nitrogen, and redissolved with 1 ml isotonic phosphate buffer, or HSA solution, human plasma, rabbit plasma and serum, respectively. The tubes were gently shaken at room temperature for 2 h to dissolve clozapine. Each sample was divided into two 500 μl aliquots; one was analyzed for total or unbound clozapine concentration by direct injection into the CE system using CE-FA method, the remaining aliquot was used for preparation of respective ultrafiltrate which was for assay of unbound clozapine concentration by ultrafiltration method.

2.6. Determination of unbound clozapine by CE-FA

Uncoated fused silica capillary (65 cm × 75 μm i.d., effective length of 35 cm) was filled with phosphate buffer (pH 7.4, containing 0.5 mol l^{-1} glycine and 1 mmol l^{-1} EDTA). The column temperature for separation was maintained at room temperature. The UV detector was set at 214 nm. The sample solutions were introduced into the separation capillary using a hydrostatic method with a height of 0.11 m for 30 s. The injection end of the capillary was then immersed into the running buffer and a voltage of 6 kV was applied between both ends. The capillary was cleaned between runs by running 30 mmol l^{-1} SDS for 8 min, and the buffer for another 8 min. The unbound concentrations of clozapine were determined by comparing the plateau height of the equilibrated samples with that of neat clozapine solution.

2.7. Determination of unbound clozapine by ultrafiltration

Ultrafiltration using Microcon Centrifugal Filter Device with 10,000 MW cut-off (Millipore, USA) was employed to determine the unbound drug concentration. Membranes were pre-treated by centrifuging 0.2 ml of 0.1 mol l^{-1} sodium hydroxide followed by rinsing with double distilled water (0.5 ml), and then the sample solution of 500 μl was transferred into the sample reservoir, equilibrated for 10 min, and centrifuged at 2000 × g with a fixed angle rotor for 8 min at room temperature. The ultrafiltrate of about 100 nl was injected with a height of 0.11 m for 30 s and analyzed by capillary electrophoresis described above. The plateau height as the measure of clozapine concentration in the ultrafiltrate was compared with that of standard solution. Possible absorption of clozapine to the filter was investigated by filtration of a solution in buffer at 50 μmol l^{-1}. None of the investigated clozapine was absorbed to the filter.

2.8. Data analysis

All statistical analyses were performed using the software package SAS (Version 8.0, SAS Institute Inc. Cary, NC, USA.). Comparisons of the means between the two methods were performed by the paired t-test and an associated $P < 0.05$ was considered significant. An ANOVA test was also used to compare the mean values of unbound clozapine concentration in different sample solutions.

3. Results and discussion

3.1. Electropherogram characteristics

The effect of sample injection time was investigated. 200 μmol l^{-1} clozapine and 550 μmol l^{-1} HSA mixed solution was analyzed for different injection time (15, 20, 25, and 30 s). While the clozapine peak width became broader with increasing injection time, the plateau height was almost unchanged regardless of the injection time, and then the injection was set at 30 s.

Typical electropherograms obtained under the conditions that were found to optimize the isolation of clozapine from plasma, serum and their ultrafiltrate are shown in Fig. 2. Matrix-specific interfering peaks were not observed. The migration time for clozapine was between 7.5 and 8.5 min. The total electrophoretic run time was 11 min.

Fig. 2(a) shows the blank electropherogram of rabbit plasma; Fig. 2(b) shows the electropherogram of clozapine in protein-free sample solution, and hence the plateau height represents the total drug concentration; Fig. 2(c), the electropherogram of clozapine in 550 μmol l^{-1} HSA solution; Fig. 2(d) and (e) show the electropherograms of 200 μmol l^{-1} clozapine in rabbit plasma and serum, respectively, and Fig. 2(f) and (g) show the electropherograms of their ultrafiltrate; Finally, Fig. 2(h) is the comparative electropherogram of clozapine in 550 μmol l^{-1} HSA solution, rabbit serum and its ultrafiltrate. It is obvious that under any condition clozapine was separated well from the other peaks due to endogenous substances, and gave a clear and wide plateau.

3.2. Effects of the separation voltage and compositions of running buffer on the plateau

The width, the shape and the position of the peaks obtained by CE-FA may be affected by the components of the running buffer and separation voltage applied. The effects on the plateau are shown in Fig. 3. Fig. 3(a) and (c) show the electropherograms of 200 μmol l^{-1} clozapine in rabbit plasma solution with separation voltage of 10 and 6 kV, respectively. It is observed that resolution between the peaks of unbound clozapine and endogenous substance decreased and the peak became narrower with an increase in applied voltage. Fig. 3(c) and (b) show the electropherograms of 200 μmol l^{-1} clozapine in rabbit plasma solution with running phosphate buffer containing 0.5 mol l^{-1} glycine and 1 mmol l^{-1} EDTA and neat phosphate buffer without glycine and EDTA. As for the former, the plateau region in the electropherogram was not flat; the shape of plateau region was significantly disturbed due to the adsorption of proteins to untreated fused silica capillary. On one hand, glycine contains amino group, which could reduce protein adsorption on the capillary wall by saturating sites on the surface that otherwise interact with proteins [17], in addition, glycine has low conductivity; On the other hand, EDTA can effectively prevent proteins from denaturation. The adsorption of analytes, especially proteins, to untreated fused silica capillary is a problem in the protein binding study, which has been overcome by addition of glycine in the running buffer in the present study. Furthermore, it is reported that a better drug plateau could be achieved by using external air pressure to improve the throughput, prevent protein loss [18].

3.3. Precision and reproducibility

Rabbit plasma and serum, human plasma were spiked with 200 μmol l^{-1} clozapine, respectively. The obtained samples were analyzed in triplicate by using the present CE system.

Results obtained by CE-FA method are compared with those from ultrafiltration techniques for clozapine. The precision and reproducibility of the method under the optimum condition are shown in Table 2. The mean values of unbound clozapine measured with CE-FA were not significantly different from those determined by ultrafiltration techniques ($P > 0.05$). This agreement indicates the reliability of the CE-FA method. The within- and between-day precisions (R.S.D.) were within 3% for both methods.

The unbound concentrations of clozapine in 550 μmol l^{-1} HSA, human plasma, rabbit plasma and serum determined by CE-FA method were 181.8±2.6, 29.6 ± 0.7, 31.4 ± 0.4, and 32.2 ± 0.5 μmol l^{-1}, respectively (Table 2). The unbound concentration of clozapine in 550 μmol l^{-1} HSA was distinguishable ($P < 0.0001$) from that in human plasma, rabbit plasma and serum (about six-fold higher than that in plasma or serum sample), indicating that HSA was not the main contributor to protein binding for

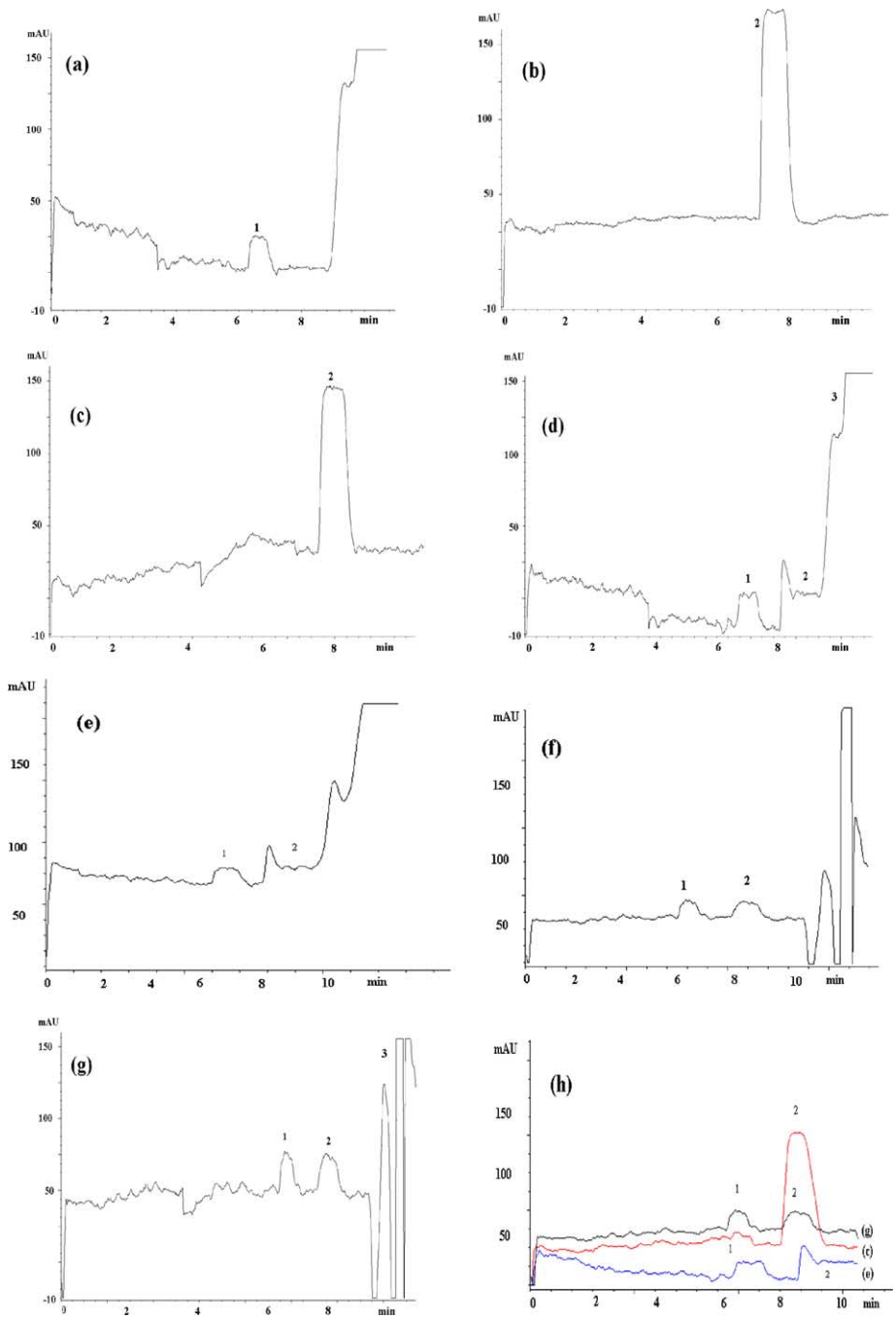

Fig. 2. (a) Electropherograms of blank rabbit plasma; (b) 200 μmol l^{-1} neat clozapine; (c) 200 μmol l^{-1} in 550 μmol l^{-1} HSA; (d) 200 μmol l^{-1} clozapine in rabbit plasma; (e) 200 μmol l^{-1} clozapine in rabbit serum; (f) ultrafiltrate of 200 μmol l^{-1} clozapine in rabbit plasma; (g) ultrafiltrate of 200 μmol l^{-1} clozapine in rabbit serum; and comparison between (c), (e) and (g) (h). The 67 mmol l^{-1} phosphate buffer (pH 7.4 containing 1 mmol l^{-1} EDTA and 0.5 mol l^{-1} glycine). Capillary length 0.65 m (0.35 m to the detector), detection 214 nm, 6 kV, 30 s hydrostatic injection (0.11 m height). 1 endogenous substance, 2 unbound clozapine.

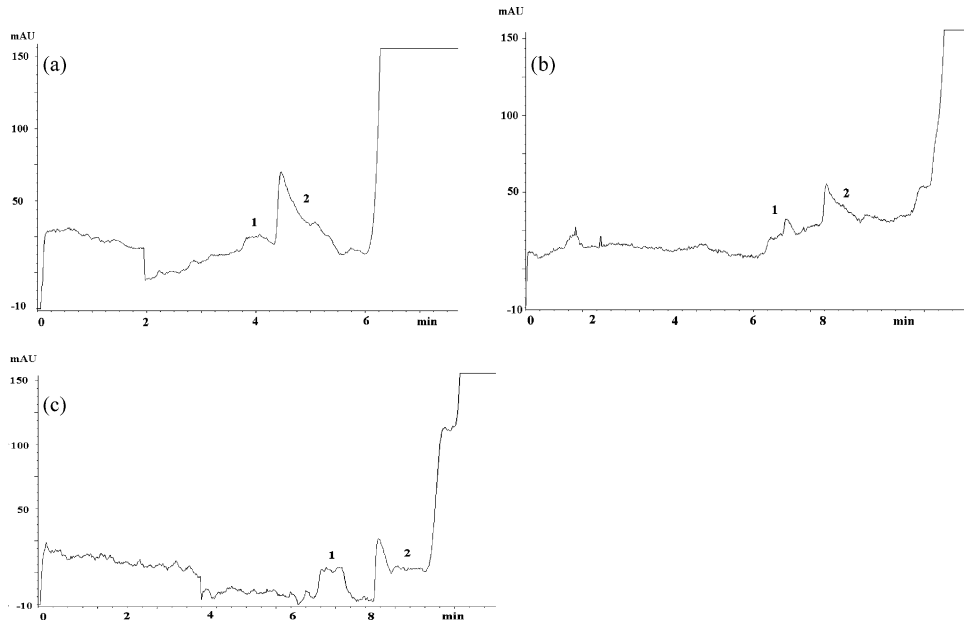

Fig. 3. Electropherograms of 200 μmol l^{-1} clozapine in rabbit plasma solution. Separation voltage: (a) 10 kV, (b) 6 kV, (c) 6 kV; running buffer: (a) phosphate buffer, 67 mmol l^{-1} (pH 7.4, containing 1 mmol l^{-1} EDTA and 0.5 mol l^{-1} glycine), 10 kV; (b) neat phosphate buffer, 67 mmol l^{-1} (pH 7.4, without EDTA and glycine), 6 kV; (c) phosphate buffer, 67 mmol l^{-1} (pH 7.4, containing 1 mmol l^{-1} EDTA and 0.5 mol l^{-1} glycine), 6 kV. Capillary length 0.65 m (0.35 m to the detector), detection 214 nm, 30 s hydrostatic injection (0.11 m height); 1 endogenous substance, 2 unbound clozapine.

Table 2
Unbound clozapine in plasma and serum measured using FA-CE and ultrafiltration[a]

Sample solution 200 μmol l^{-1}	Concentration determined by FA-CE (μmol l$^{-1)}$		Concentration determined by ultrafiltration (μmol l^{-1})
Clozapine	Within-day	Between-day	
In rabbit plasma	31.4 ± 0.4	31.9 ± 0.9	30.1 ± 0.3
In rabbit serum	32.2 ± 0.5	32.7 ± 0.8	31.2 ± 0.4
In human plasma	29.6 ± 0.7	31.1 ± 0.9	28.8 ± 0.3
In 550 μmol l^{-1} HSA	181.8 ± 2.6	185.3 ± 4.1	178.6 ± 1.8

[a] Data expressed as mean ± S.D. ($n = 3$).

clozapine in plasma or serum and other plasma proteins apart from albumin may be involved in the binding process. The unbound concentration of clozapine in rabbit plasma was similar to that obtained in rabbit serum ($P > 0.05$, ANOVA), but both were different from that in human plasma ($P < 0.05$, ANOVA).

The present CE-FA method is particularly advantageous for basic drugs whose migration behavior is similar to clozapine under a physiological phosphate buffer (67 mmol l^{-1}, pH 7.4 containing 0.5 mol l^{-1} glycine, and 1 mmol l^{-1} EDTA) as running buffer condition.

One major limitation of this method is relatively poor sensitivity. Since the internal diameter of the capillary used in CE-FA method is 75 μm, detection in such a small effective path length is always a challenge.

4. Conclusion

In conclusion, trials searching for the optimum condition were made and it was found that lower applied voltage and the additives of running phosphate buffer

were beneficial for separating the clozapine peak from the other peaks due to endogenous substances.

The results indicate that clozapine hardly binds to HSA (bound fraction was found 9.1%) and other plasma proteins apart from albumin may be involved in the binding process (average bound fraction in serum or plasma was found about 84%).

This study demonstrates the utility of CE-FA method in determination of unbound concentration in multiple equilibria system. The advantages in terms of time, reagent and the extreme simplification of the analytic procedure will make the present method particularly suitable to applications in the therapeutic drug monitoring (TDM) laboratory in the future.

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