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Short communication

Capillary electrophoretic separation of drug enantiomers in human serum

Atsushi Taga [a], Yingxiang Du [a,b], Shigeo Suzuki [a], Susumu Honda [a,*]

[a] *Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, 577-8502 Higashi-Osaka, Japan*
[b] *Department of Analytical Chemistry, China Pharmaceutical University, 24 Tonjiaxiang, Nanjing, China*

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Abstract

Enantiomers of various solutes including several basic drugs and α-amino acids were analyzed by capillary electrophoresis in diluted human serum, and chloroquine and tryptophan were found to be well enantioseparated. In order to specify the protein responsible for enantioseparation, these drug enantiomers were analyzed in the presence of various serum protein fractions. The results indicated that albumin fraction caused enantioseparation but the α and β-globulin mixed fraction, the γ-globulin fraction and the α_1-acid glycoprotein fraction did not exhibit any enantioseparation. The association constants between these drugs and albumin were roughly estimated based on our method. Approximate values were 1.50×10^3 and 1.85×10^3 M^{-1} for chloroquine enantiomers, and 1.51×10^4 and 2.45×10^4 M^{-1} for tryptophan enantiomers. The difference of the association constant values between the enantiomers was found to be 19% for chloroquine and 38% for tryptophan, when calculated based on the slower moving enantiomers.

Keywords: Enantioseparation; Drug–serum albumin binding; Human serum albumin; Chloroquine; Tryptophan

1. Introduction

The presence of enantiomers in many synthetic drugs has posed a problem for their therapeutic use, because the enantiomers often exhibit different activities to the host. For this reason the importance of simultaneous analysis of enantiomers has been recognized and various challenges have been made for this most difficult problem in analytical chemistry. Chromatographic and electrophoretic methods have greatly contributed to enantiomer analysis, and numerous reports and reviews on these methods have been published. The thematic issue of Ref. [1] is especially notable, because it collects excellent reviews on enantioseparation of drugs by leading researchers in various areas of separation science.

* Corresponding author. Tel.: +81-6-6721-2332x3811; fax: +81-6-6721-2353
E-mail address: shonda@phar.kindai.ac.jp (S. Honda).

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Various kinds of compounds ranging from small molecules to macromolecules have been shown to be effective as chiral selectors for enantioseparation of drugs. Among them proteins are attractive, because they are the principal constituents of the tissues and body fluids in the host and their structures are diverse. In the investigation of the enantioseparation of drugs by proteins high performance liquid chromatography (HPLC) has played an important role. In such studies a target protein was immobilized to a solid phase and a mixture of drug enantiomers was applied to a column packed with the thus protein-immobilized solid phase. The enantiomers could be separated from each other provided suitable elution conditions were realized. The significant enantioselectivity reported for various proteins was reviewed by Haginaka [2]. Capillary electrochromatography has also contributed to the enantioseparation of drugs [3], though the driving force was not the pressure flow as in HPLC but mainly the electroosmotic flow (EOF). Analysis by this method is performed in free solution without any solid phase unlike in HPLC and CEC, and therefore does not need immobilization of proteins. Analysis can be performed simply by introducing a sample solution of drug enantiomers to a running buffer containing a protein (affinity capillary electrophoresis, ACE). The enantiomers can be separated from each other under suitable conditions. The proteins examined for HPLC have also been tested for CE and proved effective for enantioseparation of various drugs [4]. In addition, it is expected that the magnitudes of interaction of both enantiomers can simultaneously be estimated from their migration behavior based on the theory developed by our [5,6] and Whiteside's [7,8] groups. Along with the ordinary type CE, CE based on the frontal analysis mode developed by Nakagawa's group [9,10], is also useful for enantiomer analysis. This method allows estimation of the proportion of unbound enantiomers and thereby the magnitude of interaction. However, it requires analysis of each enantiomer to obtain the proportion.

Thus, there have been a number of works on the enantioseparation of drugs using proteins as chiral selectors, but the aim of these works has mostly been either in the demonstration of the usefulness of these proteins as chiral selectors or in the elucidation of the mechanism of such enantioselective interactions. The enantioseparation of drugs with albumin and α_1-acid glycoprotein is especially interesting, because they are the component proteins of blood, the enantioseparation of drugs in blood will also provide much more straightforward information on the different activities of drug enantiomers to the host, though there have been only a few examples of such attempt. In this paper, we report successful examples of enantioseparation of one basic drug and one alpha amino acid in human serum and an attempted specification of the protein responsible for this enantioseparation.

2. Experimental

2.1. Materials

Primaquine diphosphate (PRI) was purchased from Aldrich Chemical Company (Milwaukee, WI). Chloroquine diphosphate (CHL), propranolol hydrochloride (PRO), doxylamine succinate (DOX), salbutamol hemisulfate (SAL), and tryptophan methyl ester (TRY-ME) were from Sigma Chemical Company (St. Louis, MO). Phenylalanine (PHE) and tryptophan (TRY) were from Wako Pure Chemicals (Chuo-ku, Osaka, Japan). All these solutes were obtained as racemic mixtures and used as obtained. The authentic specimens of *R*- and *S*-enantiomers of tryptophan were also obtained from Wako. All chemicals used for preparation of running buffer and capillary coating were of the highest grade commercially available. Deionized and glass-ware distilled water was used for the preparation of running buffers and reagent solutions.

Heparinized, pooled human serum was obtained from BIO Whittaker (Quakersvill, MD). Serum protein fractions were purchased from Sigma and used without further fractionation.

2.2. Apparatus

An Applied Biosystems model 270A apparatus was used for the enantioseparation of drugs, which

was composed of a power supply, an injection device based on vacuum introduction, a capillary oven thermostated by air circulation, and a UV detector. Analysis was preformed at 30 ± 0.1 °C, and peaks were recorded and processed by a Hitachi D-2500 integrator.

2.3. Coating of capillaries

A piece of a 75 μm i.d.-fused silica capillary (Polymicro Technologies, Phoenix, AZ) was installed on the CE apparatus after a detection window was created at the 20-cm position from the outlet. It was rinsed with 1.0 M sodium hydroxide followed by water (each for 15 min) to refresh the inner wall. The refreshed capillary was coated dynamically with hexadimethrine bromide (Polybrene) by flushing a 5.0% aqueous Polybrene solution for 20 min, to make the first layer. The capillary was then rinsed with water for 15 min and flushed with a 3.0% aqueous solution of polyacrylic acid for 20 min, to make the second layer. Thus prepared double coated capillary was rinsed with water and finally equilibrated with a running buffer to be used.

2.4. Running buffer

Phosphate buffer of 50 mM at pH 6.8, was used throughout this work. A human serum sample obtained as above was diluted to various concentrations with this phosphate buffer, and the diluted solutions were passed through a membrane filter (pore size, 0.45 μm) and used as running buffers.

2.5. Ka estimation

This was done basically according to our previous paper [6] using the slope and the ordinate intercept of the straight line prepared by the $(t - t_1)^{-1}$ vs. $[C]^{-1}$ plot, where t and t_1 are the migration times of a drug enantiomer in the presence and the absence of serum, respectively, and $[C]$ is serum concentration.

3. Results and discussion

3.1. Coating of capillary for the prevention of protein adhesion

CE has been used for the analysis of various kinds of substances, but as far as the analysis involving proteins is concerned, there have been a serious problem of their adhesion on the capillary inner wall, since the capillary material has been exclusively fused silica having the silanol group. This problem has been almost solved by coating with linear polyacrylamide, but this coating material is not stable and durable to alkaline solutions. Katayama et al. [11] proposed dynamic double coating with Polybrene and an acidic polysaccharide and demonstrated the stability of double coated capillary. We have extended this technique to other cationic/anionic polymer couples. In the present work, we used Polybrene/polyacrylic acid double coated capillaries [12], which have also been proven very stable and durable. In this coating almost all silanol groups were first coated with Polybrene, to which polyacrylic acid was bound. The capillary inner wall was negatively charged stronger than in uncoated fused silica capillary. The reproducibility of analysis was also high over a wide pH arrange [12].

3.2. Selection of separation conditions

In the present work, we aimed at obtaining the basic information on the difference in the magnitude of interaction between drug enantiomers with a protein in blood, which has normally a pH range of 6.5–7.5. Therefore, a running buffer having its pH value in this range should be used. So in this paper, we report the result obtained with 50 mM phosphate buffer adjusted to pH 6.8.

The analytes adopted were enantiomers of several basic drugs and a few α-amino acids described in Subsection 2.1, which were only slightly protonated at this neutral pH. Since the capillary inner wall had negative charge, EOF was toward the cathode. Therefore, the drug enantiomers moved to the cathode almost exclusively by EOF, to give their peaks at around 5 min.

3.3. Separation of the enantiomers of basic drugs and α-amino acids

In contrast to the reference experiments mentioned in Section 3.2, the peaks of the basic drugs were retarded to 10–15 min. Baseline showed marked drift presumably due to pH change across the capillary, when serum sample was added to the running buffer. The peaks of the α-amino acids were much more retarded to 15–25 min. The electropherograms of PHE and TRY showed the presence of a deep trough at ca. 20 min, presumably because these amphoteric solutes changed ionic state during migration. Although most of these analytes gave single peaks without enantioseparation, CHL (Fig. 1) and TRY (Fig. 2) were exceptions, giving double peaks due to the enantiomers.

This enantioseparation became more prominent as the concentration of added serum sample increased. α: 1.00 (not separated) (CHL), 1.01 (TRY) at 10 v/v%; 1.02 (CHL), 1.05 (TRY, at 20 v/v%. Rs: 0.00 (not separated) (CHL), 0.33 (TRY) at 10 v/v%; 0.33 (CHL), 1.45 (TRY) at 20 v/v%. For TRY the *R*-enantiomers moved faster than the *S*-enantiomer (peaks were assigned by co-migration with the authentic specimens), but for CHL the peaks could not be assigned to the enantiomers because neither of the authentic specimens of *R* and *S* enantiomers were commercially available.

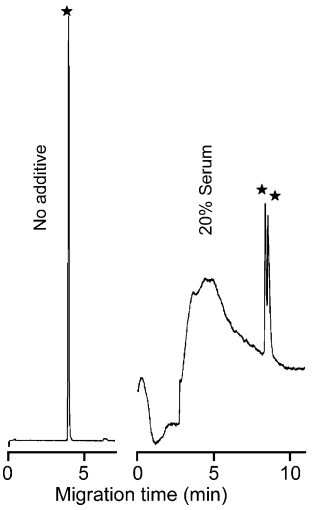

Fig. 1. Enantioseparation of CHL in a buffer containing human serum. Capillary, Polybrene/polyacrylic acid double coated (75 μm i.d., 50 cm); electrophoretic solution, 50 mM phosphate buffer (pH 6.8) containing serum equivalent to 20 v/v%; capillary temperature, 30 °C; applied voltage, 10 kV; sample introduction, from the anodic end; detection, UV absorption at 230 nm.

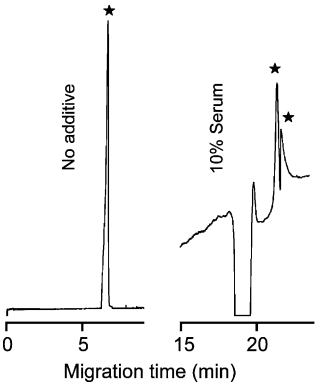

Fig. 2. Enantioseparation of TRY in a buffer containing human serum. The analytical conditions as in Fig. 1, except that serum concentration was 10 v/v%.

The relative standard deviation of migration time for CHL enantiomers was 0.94% (1st peak)/0.95% (2nd peak) and that for TRY enantiomers was 4.4.%(1st peak)/4.3% (2nd peak), for five repeated measurements. Thus, relatively high reproducibility was obtained, because of less adhesiveness of serum proteins to this double coated capillary than to non-treated capillary. The double coated capillary was durable and could be used at least 20 times without marked change of migration time. Treatment with 0.1 M sodium hydroxide followed by 0.3 w/v% polyacrylic acid for 2 min was effective to restore the original state and lengthen column life. This treatment was essential when running buffers containing more than 15% serum was used.

3.4. Magnitude of interaction

In the ACE mode of CE performed in free solution, the migration time change of a sample

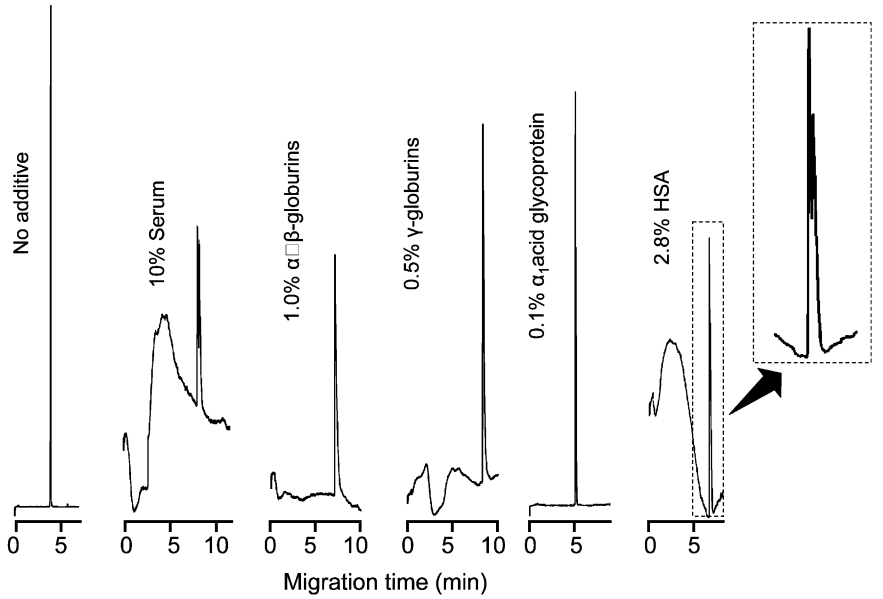

Fig. 3. Specification of serum protein fractions for enantioseparation of CHL. The analytical conditions as in Fig. 1.

depends on the concentration of an additive in running buffer. The authors group studied the relationship between migration time change and additive concentration, and established a method for estimating association constant [5,6]. It is based on the theory that the association constant Ka can be expressed as $(B\ t_1+1)/A\ t_1$, where A and B are the slope and the ordinate intercept, respectively, of the straight line, prepared by the double reciprocal plot of migration time change of

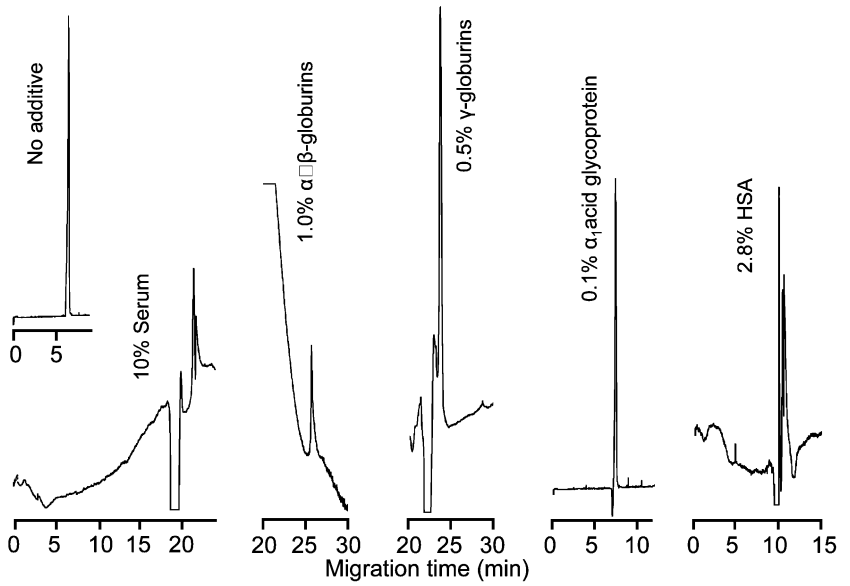

Fig. 4. Specification of serum protein fractions for enantioseparation of TRY. The analytical conditions as in Fig. 2.

sample (a drug enantiomer in this case) between the presence and the absence of the additive (a serum protein in this case) vs. the additive concentration [C], respectively, and t_1 is the migration time of the sample in the absence of the additive. With respect to CHL the tentative values of A and B were 1.04×10^{-3} and 1.33, respectively, for $C = 0.50$–3.0 w/v% (7.6×10^{-5}–4.5×10^{-4} M), and thereby Ka could be calculated as 1.50×10^{3} M^{-1} for the faster moving enantiomer. The corresponding values for the slower moving enantiomer were $A = 1.01 \times 10^{-3}$, $B = 1.64$, $[C] = 0.50$–3.0 w/v% (7.6×10^{-5}–4.5×10^{-4} M), and Ka $= 1.85 \times 10^{3}$ M^{-1}. With respect to TRY, A, B, [C], and Ka were 1.37×10^{-4}, 1.92, 0.10–1.5 w/v% (1.5×10^{-5}–2.3×10^{-4} M), and 1.51×10^{4} M^{-1}, respectively, for the faster moving enantiomer, and 7.16×10^{-5}, 1.81, 0.10–1.5 w/v% (1.5×10^{-5}–2.3×10^{-4} M), and 2.45×10^{4} M^{-1}, respectively, for the slower moving enantiomer. These results indicate that the difference of Ka values between the enantiomers was 19% for CHL and 38% for TRY of the slower moving peaks.

3.5. Identification of the protein responsible for enantioseparation

The results shown in Figs. 1 and 2 mean that there was at least more than one protein in the serum sample that interacted differently with the enantiomers for CHL and TRY. However, these figures give no information on the specification of the protein. For the specification of the protein, analysis by the normal system (protein as sample—ligand as buffer additive, [5]) is considered to be most useful. Therefore, serum was analyzed in a buffer containing either CHL or TRY, but unfortunately the addition of high concentrations of drugs resulted in baseline increase at the wavelengths of their absorption maxima, which hampered detection of separated proteins. Therefore, we adopted the second best strategy, namely comparative analysis of drug enantiomers in various serum protein fractions. Since in this strategy analysis should be carried out under similar conditions as in blood, this was done using the reported value of the average proportion of each fraction [13]. The results obtained for CHL and TRY are shown in Figs. 3 and 4, respectively.

For both drugs enantioseparation was observed in the albumin fraction, but not in the mixed fraction of α and β-globulins, the γ-globulin fraction, and the α_1-acid glycoprotein fraction, a result consistent with the general knowledge on drug binding to blood [14]. The enantioselectivity of the albumin fraction (CHL: $\alpha = 1.02$, Rs $= 0.50$; TRY: $\alpha = 1.02$, Rs $= 0.35$) was approximately to the same extent as that observed for the whole serum. Although existence of other proteins, which might interact with these solutes cannot theoretically be excluded, Figs. 3 and 4 provide strong evidence for the contribution of albumin.

Comparison of the CHL peaks for serum and HSA (Fig. 1) indicates that the former is slightly slower than the latter. With respect to the TRY peaks the serum-containing buffer gave much slower peak than the HSA-containing buffer (Fig. 2). Such velocity difference implies that any other components than HSA might have interacted, even though not enantioselectively, with these solutes, affecting Ka estimation to some extent.

4. Concluding remarks

Enantioseparation was achieved for a basic drug (CHL) and an α-amino acid (TRY) out of eight candidates using a neutral phosphate buffer containing human serum. Double coating of the capillary inner wall by Polybrene, followed by polyacrylic acid was an important factor that allowed this enantioseparation. We might be able to find out other conditions for enantioseparation possibly by adding certain kinds of compounds to running buffer. However, addition of such compounds should be avoided, because it will make estimation of association constant more difficult. On the other hand the proposed ACE method enabled simultaneous estimation of association constants to a serum protein, for both enantiomers. The applicability of the ACE method to enantioseparation and simultaneous association constant estimation demonstrated in the present

paper will be further extended to a wider range of analytes of biomedical interest.

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