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Determination of the binding of a β_2-blocker drug, frusemide and ceftriaxone to serum proteins by capillary zone electrophoresis

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Abstract

A modified Hummel–Dreyer method was used to study the binding of drugs with serum proteins by high performance capillary electrophoresis. The study was carried out to check the possible interaction between serum proteins and a highly selective β_2-blocker, ICI 118551 (ICI). To prove the suitability of the method the protein binding of frusemide and ceftriaxone, drugs previously investigated, was also studied. The analyses were carried out by injecting a solution of $s\alpha_1$-acidic glycoprotein (α_1-AGP) or human serum albumin in 70 mM NaH_2PO_4/Na_2HPO_4(pH 7.4) buffer into an uncoated fused silica capillary filled with the same buffer. In the capillary, maintained at a working temperature of 35°C, a known amount of the ICI, frusemide or ceftriaxone was added. The method allows the bound drug to be determined directly.

Keywords: Ceftriaxone; Capillary zone electrophoresis; Drug–protein complexes; Frusemide; Hummel–Dreyer method; ICI 118551

1. Introduction

A new drug can be correctly used in therapy if the possible protein binding of the drug to serum proteins is studied. It is generally accepted that plasma binding of drugs is a transport mechanism that also acts as protection against metabolism to prolong the duration of drug activitiy [1]. Binding plays an important role in drug availability [2–4] because the free drug alone is considered to diffuse from the blood to the extravascular action site and exhibit pharmacological activity. Therefore an analytical method which determines the extent of binding of a drug to serum macromolecules should always be included in its analytical profile.

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In two previous papers [5,6] the chiral resolution of a new β-blocker, ICI 118551 (ICI), and the determination of its related compounds was reported. To complete the study of this molecule it was decided to look for a suitable method to determine the size of the probable bond between ICI and serum proteins. As stated above, the determination of the binding provides useful information about the pharmacological activity, biological distribution and clearance of the drug. ICI, one of the first selective β_2-blockers synthesized [7,8] is the racemic mixture of the erythro form of 1-[(2,3-dihydro-7-methyl-1*H*-inden-4-yl)oxyl]-3-[(1-methylethyl) amino]-2-butanol. ICI is intended for use in the treatment of somatic problems related to anxiety states.

In the binding studies the serum protein most likely to interact with the drug must be considered. Since ICI is a basic drug it can be supposed that it binds with sα_1-acidic glycoprotein (α_1-AGP). Therefore it was investigated whether ICI binds with this protein and if one or more binding sites are involved.

Many different analytical methods, such as ultrafiltration [9,10], gel filtration [11,12], equilibrium dialysis spectroscopy [13] and chromatography [14–20] have been used for this purpose. Dialysis and ultrafiltration have been the most frequently used techniques because they provide accurate measurements of binding parameters with inexpensive equipment even if it can be very laborious to acquire a large number of data points. A few years ago an original chromatographic method, high-performance frontal analysis (HPFA), was suggested [21] and more recently capillary electrophoresis procedures have been used [22–25].

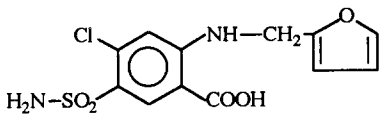
In the authors' opinion the most suitable methods for determining the bound and unbound drug concentrations are those that involve a limited number of protein–drug complex equilibria and require only a small amount of drug. For this reason ICI 118551 have been analysed using capillary electrophoresis by modifying the well known Hummel–Dreyer method [26].

The validity of the proposed method was checked by determining the binding of frusemide (furosemide) and ceftriaxone with human serum albumin (HSA) [27]. The chemical structures of frusemide, ceftriaxone and ICI 118551 are shown in Fig. 1.

2. Experimental

2.1. Reagents and chemicals

Frusemide and ceftriaxone, HSA (fraction V) and human glycoprotein α_1-acid (purified from Cohn fraction VI) were purchased from Chemical Sigma Co., Italian Division (Milan, Italy). A pure standard of ICI 118551 was kindly supplied by Italian Zeneca Pharmaceutical Division (Milan, Italy). Sodium hydroxide and the phosphate salts



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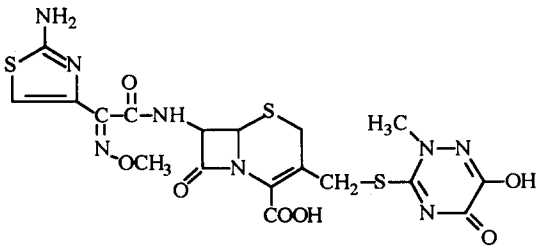

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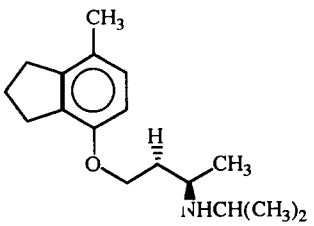

III

Fig. 1. Chemical structures of frusemide (**I**), ceftriaxone (**II**) and ICI 118551 (**III**).

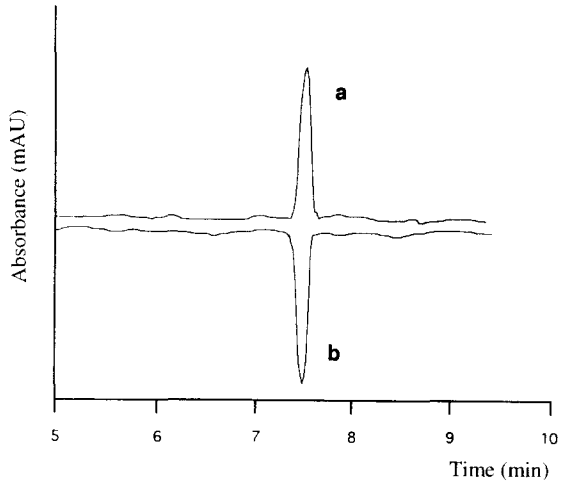

Fig. 2. (a) Electropherogram obtained by injecting frusemide (0.004 mg ml^{-1}) into an uncoated capillary filled with 70 mM phosphate buffer (pH 7.4). (b) Electropherogram obtained by injecting 70 mM phosphate buffer (pH 7.4) into the same capillary filled with 70 mM phosphate buffer (pH 7.4) containing frusemide (0.004 mg ml^{-1}). Electrophoretic conditions: injection time, 2.5 s; λ, 260 nm; voltage, 10 kV; 55 μA; temperature, 35°C.

were of analytical-reagent grade; water was of HPLC grade (Merck, Darmstadt, Germany).

2.2. Equipment

The electrophoretic experiments were carried out with a Spectraphoresis 1000 apparatus (Thermo Separation Products, San José, CA) equipped with a multiwavelength UV–Visible detector (SpectraFocus) with a deuterium lamp and cooling-air circulation using Peltier effect system (15–60°C).

The capillary electrophoresis was controlled and the data were evaluated with the SpectraPhoresis CE v. 1.05 B software (Thermo Separation Products).

The separation of compounds of interest was performed in a bare fused-silica capillary (Supelco, Bellefonte, PA) of 50 μm i.d. and length 42 cm (effective length 34 cm).

2.3. Capillary zone electrophoresis (CZE) conditions

Capillary conditioning was done each day (washing with 0.1 M sodium hydroxide for 30 min) and the subsequent conditioning cycles were done before each run (washing with water for 10 min and with buffer for 3 min).

CZE was performed in a 70 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.4, adjusted with 1 M NaOH) at a working temperature of 35°C.

The samples were applied by a vacuum of 2.5 s ($\approx$3.5 nl s^{-1}). The temperature of the capillary cartridge was maintained at 35°C.

2.4. Drug–protein complex analysis

2.4.1. Calibration curves

The calibration curve of each drug was obtained by filling the capillary with the buffer containing different and increasing amounts of drugs and

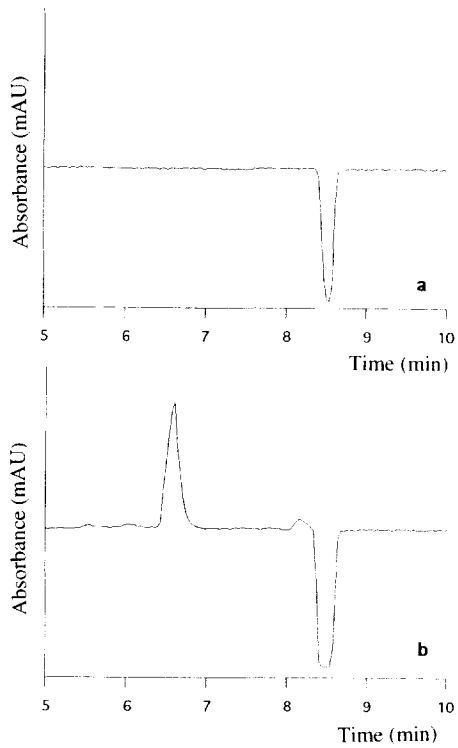

Fig. 3. Electropherograms obtained by injecting: (a) 70 mM phosphate buffer (pH 7.4); (b) 0.055 mM HSA. Electrophoretic conditions: capillary filled with 70 mM phosphate buffer (pH 7.4) containing ceftriaxone (0.04 mg ml^{-1}); injection time, 2.5 s; voltage 10 kV; current, 55 μA; temperature, 35°C.

injecting a fixed volume of phosphate buffer. By plotting area values against concentrations a linear response was obtained in the concentration ranges: frusemide 0.001–0.1 mg ml^{-1}; ICI 118551 0.001–0.01 mg ml^{-1}; and ceftriaxone 0.008–0.04 mg ml^{-1}.

2.4.2. Sample preparation

The protein, HSA or α_1-AGP, in two different concentrations (0.011 or 0.055 mM) was dissolved in the phosphate buffer.

2.4.3. Binding determination

The analyses were carried out by injecting the protein solution, prepared as described above, in the running buffer containing a known amount of drug.

3. Results and discussion

The presence of an absorbing compound in the running background electrolyte (B.G.E.), such as one of the tested drugs, caused a strong absorbance value. Therefore any variation of the drug concentration in the B.G.E. was visualized in the electropherograms as a negative peak.

Before the investigation of the ICI 118551 protein binding, the method was tried with two drugs, frusemide and ceftriaxone, for which the HSA bindings had been previously studied by other authors using HPLC [17,27].

The analyses were all carried out using the following scheme:

First, by checking the linear correlation between the injected drug and the peak area value. For this purpose different and increasing amounts of the drug were injected into the capillary filled only with phosphate buffer.

Second, by checking the electrophoretic response by injecting a fixed volume of the phosphate buffer into the running B.G.E. containing a known amount of drug. The electropherogram showed a negative peak at the same migration time as the drug and an area which grew linearly with increasing drug concentration in the capillary (Fig. 2). The calibration curves obtained by these two methods were perfectly superimposable.

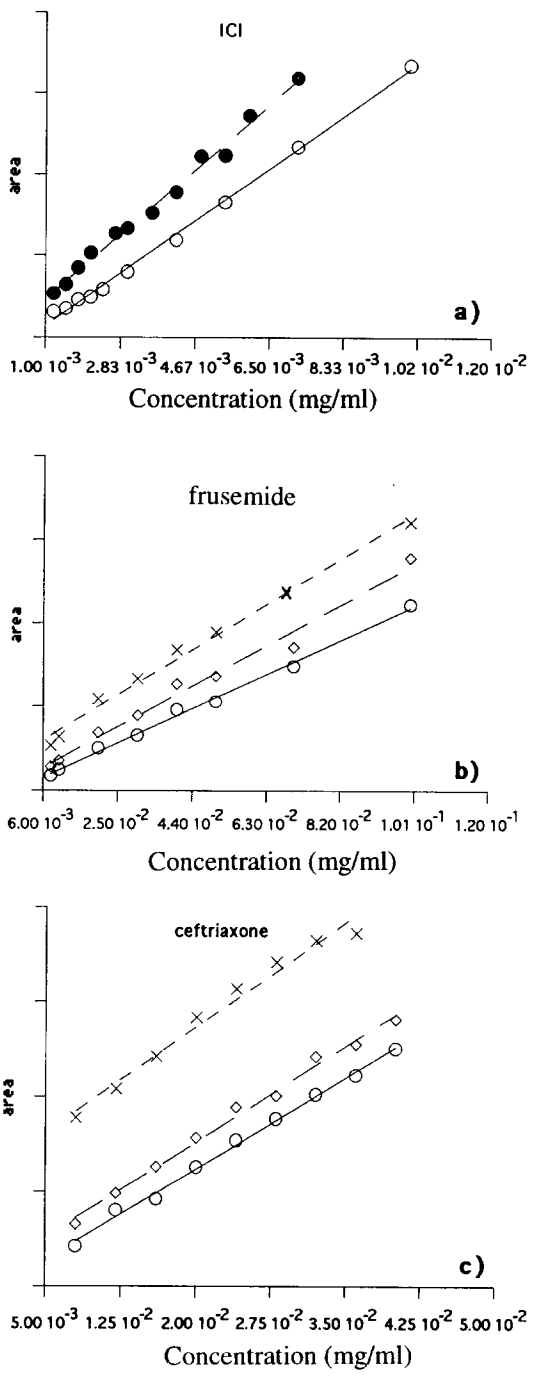

Fig. 4. Calibration and working curves obtained by injecting (○) buffer, (●) HGA, (◇) 0.011 mM HSA or (×) 0.055 mM HSA. Capillary filled with buffer and different amounts of drug: (a) ICI; (b) frusemide; (c) ceftriaxone.

Table 1
Comparison of the binding constants of frusemide and ceftriaxone obtained in this work and with different techniques

K_1	K_2	Temperature (°C)	Technique
Frusemide			
9.4×10^4	5500	35	CZE (this work)
1.68×10^5	9600	37	HPLC [16]
6.88×10^4		37	Equilibriumdialysis [30]
5.07×10^4	1.58×10^4	37	Equilibrium dialysis [31]
3×10^5	–	Ambient	Ultrafiltration [32]
Ceftriaxone			
2.3×10^5	–	35	CZE (this work)
8.06×10^4	–	37	Equilibrium dialysis

Third, by injecting a fixed amount of protein, HSA or α_1-AGP in the capillary filled with phosphate buffer containing a known amount of drug. The protein–drug complex may or may not be formed. In both cases the electropherogram shows a positive peak, related to the complex and/or to the free protein, preceded or followed by a negative peak. The negative peak represents the amount of the drug bound to the protein (Fig. 3) or, if the complex is not formed, the variation in the B.G.E. due to the injected buffer.

The peak area related to the bound drug is bigger than that corresponding to the buffer injection. The curves which display the two peak areas is the same plot are linear (Fig. 4). The upper straight line corresponds to the bound drug. The difference between the upper and lower lines is due to the bond. Suitable concentrations of frusemide or ceftriaxone in the running buffer and the protein sample were carefully studied. The concentration of the proteins, HSA or α_1-AGP, were tested in the range 0.0110–0.0550 mM while the drug concentration range was 0.001–0.3 mg ml^{-1}. The drugs gave a linear response only between 0.001 and 0.1 mg ml^{-1}. The loss of linearity at concentrations higher than 0.1 mg ml^{-1} was caused by saturation of the detector.

Classical Scatchard plots [28] were drawn using the data obtained from the binding determination of ceftriaxone and frusemide. This plot shows whether one or more protein binding sites are involved. In the simplest situation, where only one binding site is involved, the plot is linear. When two or more binding sites are involved the Scatchard plot is not a straight line. By drawing two straight lines tangential to the highest and lowest ends of the curved Scatchard plot, the binding constants of frusemide and ceftriaxone were determined and compared with those obtained by different techniques (Table 1).

The possibility of competition between frusemide and ceftriaxone, present at the same time in the running buffer, for the protein binding was also considered. The two drugs did not seem to be in competition for the HSA binding sites. Therefore, the possible interaction of ICI 118551 with human serum proteins, HSA and α_1-AGP, was studied following the scheme described above. A suitable concentration range was 1×10^{-5}–1.2×10^{-6} mM; the proteins were kept at a constant concentration of 5.5×10^{-5} mM. As expected, ICI 118551 did not bind significantly with HSA whereas a bond with α_1-AGP was evident (Fig. 5). The curved Scatchard plot clearly showed that more than one class of binding sites is involved (Fig. 6) in the binding. The two binding constants calculated for ICI were 1.12×10^5 (K_1) and 7.5×10^2 (K_2).

Inter-day and intra-day repeatabilities of the assays for frusemide, ceftriaxone and ICI 118551 were made by performing repeat analyses at high, middle and low concentrations of each drug in the running buffer. For the concentrations of the calibration curves, described in Fig. 3, the precision (RSD) of the mean value ($n = 12$ for each drug) of the negative peak area relating to the bound drug did not exceed 15%. The satisfactory reproducibility of the areas and the retention time

of the protein excludes the presence of a strong protein–capillary interaction.

The purpose of this work was to determine the possible binding of ICI 118551, a β_2-blocker, with human serum proteins, under analytical conditions as similar as possible to those of the biological system. Therefore, as in vivo, the protein–drug complex occurs when the drug (the ligand) comes into contact with the circulating human serum proteins; an attempt was made to mimic the biological system by injecting only the protein solution into the running buffer containing the drug. In the classical Hummel–Dreyer method the chromatographic column is eluted with a mobile phase containing a known amount of drug. This mobile phase has been used to prepare the sample by dissolving a known amount of protein. In the sample drug–protein complex, the free drug and the free protein were in equilibrium. A modified Hummel–Dreyer method, used to determine warfarin–HSA binding [18] by HPLC with a non-absorptive column, employed as sample a solution containing only protein whereas the mobile phase was a phosphate buffer solution containing the drug as in the classical method. In the modified method the drug–protein binding equilibrium is reached easily and requires a shorter time. However, whatever HPLC method is used to determine the drug–protein binding the equilibria need to be continually re-established since the protein–drug complex separates from the free drug during migration down the column.

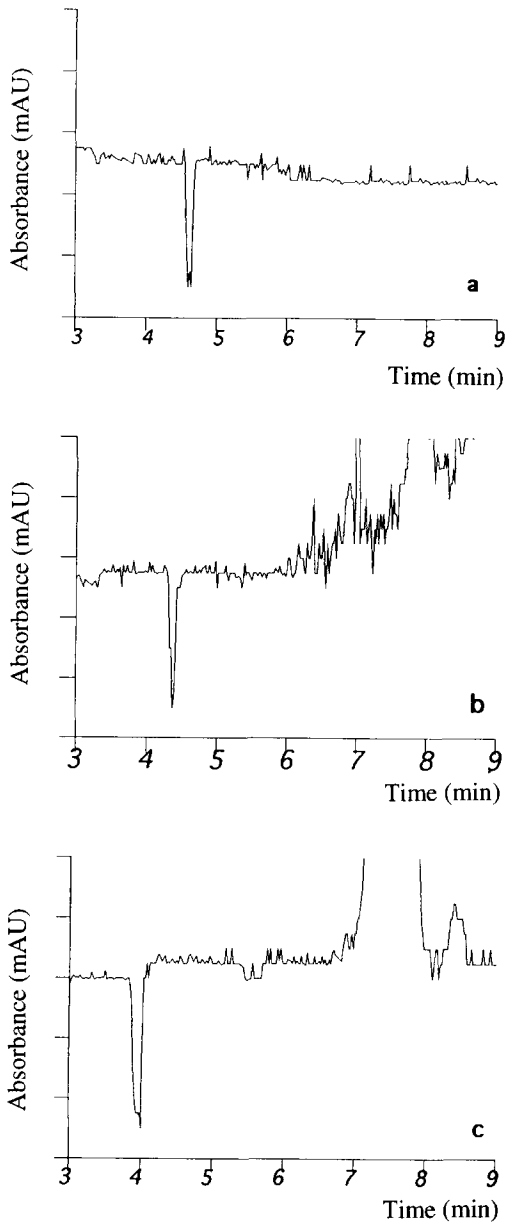

Fig. 5. Electropherograms obtained by injecting: (a) buffer only; (b) 0.011 mM HSA, (c) 0.011 mM α_1-AGP. Electrophoretic conditions: capillary filled with 70 mM phosphate buffer (pH 7.4) containing ICI (0.006 mg ml^{-1}); injection time, 2.5 s; λ, 200 nm; voltage, 10 kV; current, 55 μA; temperature, 35°C.

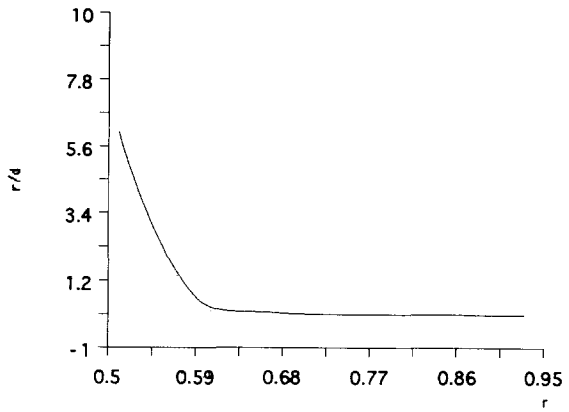

Fig. 6. Scatchard plot illustrating the binding of ICI to α_1-AGP. r = ratio of millimoles of drug bound to millimoles of HGA; d = free drug concentration.

For this reason, in the ICI 118551 protein binding determination HPCE using the modified Hummel–Dreyer method was preferred. However, binding determination with HPCE involves only one equilibrium, which occurs when the protein comes into contact with the drug in the running buffer. The absence of a stationary phase simplifies the drug–protein complex formation.

The binding constants of frusemide–HSA and ceftriaxone–HSA complexes for the primary or secondary binding sites vary significantly when compared to those obtained by HPLC; however, their values were in good agreement with those reported in the literature for human plasma binding [29]. These differences, as stressed by many authors, are due to the experimental conditions and methodology used in the determination of plasma protein binding.

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