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# On the zopiclone enantioselective binding to human albumin and plasma proteins. An electrokinetic chromatography approach

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ABSTRACT

In this work, a methodology for the chiral separation of zopiclone (ZPC) by electrokinetic chromatography (EKC) using carboxymethylated-β-cyclodextrin as chiral selector has been developed and applied to the evaluation of the enantioselective binding of ZPC enantiomers to HSA and total plasma proteins. Two mathematical approaches were used to estimate protein binding (*PB*), affinity constants ($K_1$) and enantioselectivity (*ES*) for both enantiomers of ZPC. Contradictory results in the literature, mainly related to plasma protein binding reported data, suggest that this is an unresolved matter and that more information is needed. Discrepancies and coincidences with previous data are highlighted.



## 1. Introduction

Drug action is the result of a large number of pharmacological processes that take place in the living systems. It is well known that most of these processes present a high degree of enantioselectivity and thus, the pharmacological characteristics of chiral drugs often vary between enantiomers [1]. Consequently, in such cases, there is a special interest within pharmaceutical industry to develop optically pure drugs, like eszopiclone, the S(+)-enantiomer of zopiclone (ZPC) [2], once it has been demonstrated that it shares the pharmacological properties, whereas the R(−)-enantiomer has no hypnotic activity [3].

The investigation of enantioselectivity of drugs in their binding with human plasma proteins represents a great challenge in clinical pharmacology. When plasma proteins and racemic drugs interact, two diastereomeric adducts are formed with potential differences in their protein binding which may result in different pharmacokinetic profiles for the individual enantiomers [4]. For the investigation of drug enantioselective binding, different approaches have been proposed. The majority of these methodologies include a first step in which the free drug fraction is separated from the bound drug once the drug-protein equilibrium has been reached using different techniques, such as the traditional equilibrium dialysis [5], ultracentrifugation [5] or ultrafiltration [6]. The second step includes chiral analysis of enantiomers, habitually on the unbound fraction. In this context, enantioseparation has become one of the most important fields of modern analytical and bioanalytical chemistry [7]. Different analytical techniques have been proposed for chiral drugs separation, such as high-performance chromatography (HPLC), gas chromatography, capillary electrophoresis or supercritical fluid chromatography [8].

Zopiclone is a non-benzodiazepine hypnotic drug used for treatment of insomnia. Its mechanism of action consists in binding to the $GABA_A$ channel, increasing the inhibitor effect of GABA (the same mechanism of benzodiazepines). The separation of the enantiomers of ZPC has been achieved by different chromatographic and electrophoretic methods [9–24]. Chromatographic methods included different chiral stationary phases [9–14] using fluorimetric [9,10] or UV [14] detection, beta-cyclodextrin bonded phases [15,16] or mobile phases with cyclodextrins as chiral selectors [17]. Recently, the chiral separation for the quantitation of eszopiclone using LC–MS/MS and AGP chiral column was reported [18]. Enantioseparation of ZPC in CE has been obtained upon the addition of neutral cyclodextrins as β-cyclodextrin [19,20,22], γ-cyclodextrin [23], or hydroxypropyl-β-cyclodextrins [24] to the separation buffer. The use of cyclodextrin-modified gold nanoparticles (GNPs) was also recently reported [21].

Important differences in plasma protein binding (*PB*) values for racemic ZPC (45–85% [3,9,25–28]) have been published, suggesting that this information still deserves more attention. A value

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45% is available from the Spanish Drug Agency [27] and figures in the well-known DrugBank database [28]. Only one reference provides plasma *PB* values for R- and S-ZPC (estimated at 4 °C) [9], and another one for S-ZPC (in vivo) [3]; differing in their values. Particular studies involving individual proteins are not available, except from [9]; however the reported data on *PB* to human serum albumin (HSA) (as well as α1-acid glycoprotein, AGP) are too low compared with those encountered for plasma. These results, based on equilibrium dialysis at 4 °C and HPLC with chiral columns, also include affinity constant ($K_1$) to HSA for racemic ZPC and its enantiomers, from which an estimation of enantioselectivity to HSA (not reported by the authors in terms of a concrete value) can be derived. Both, the contradictory data and the lack of contrasted information on $K_1$, particularly for ZPC enantiomers, point out the need to amplify this kind of studies in order to establish a more reliable ZPC enantioselectivity (*ES*).

In this paper, the enantioselective binding of ZPC to human serum albumin (HSA) and plasma proteins is evaluated. HSA is the most abundant protein in the circulatory system (i.e. with the largest complexation potential), moreover it exhibits the highest potential of enantiodifferentiation among the plasmatic proteins. The proposed methodology comprises the ultrafiltration of pre-equilibrated samples containing HSA (or human sera) and racemic drug and the enantiomeric resolution and analysis of the unbound fraction [6,29,30]. In order to evaluate the enantioselective binding of ZPC enantiomers, the electrokinetic chromatography (EKC)-partial filling technique (PFT) with the anionic carboxymethylated-β-cyclodextrin (CM-β-CD) as chiral selector was used. EKC-PFT, which involves the filling of a separation capillary only in part with a chiral selector, presents several advantages as the extremely low consumption of cyclodextrin, since the inlet and outlet vials of the separation system are free of chiral selector [8]. The use of a cyclodextrin as chiral selector was preferred here to HSA, used previously by our group (affinity EKC [29]), due to their broad spectrum of enantioselectivity and better electrophoretic features (signal-to-noise ratio, resolution, etc.).

## 2. Experimental

### 2.1. Instrumentation

A Beckman P/ACE MDQ Capillary Electrophoresis System equipped with a diode array detector (Beckman Coulter, Fullterton, CA, USA), and 32Karat software version 8.0 was used throughout. A 50 μm inner diameter (i.d.) fused-silica capillary with total and effective lengths of 31.5 and 21 cm, respectively, was employed. Electrophoretic solutions and samples were filtered through 0.45 μm pore size nylon membranes (Micron Separation, Westboro, MA, USA) and degassed in an ultrasonic bath (JP Selecta, Barcelona, Spain) prior to use. A Crison Micro pH 2000 pH meter from Crison Instruments (Barcelona, Spain) was employed to adjust the pH of buffer solutions.

A Selecta thermostatized bath (JP Selecta, Barcelona, Spain) was used for samples incubation. For the ultrafiltration of samples, Microcon YM-10 cellulose filters of a molecular weight of 10,000 MWCO (Millipore Corporation, Bedford, MA, USA) and a centrifuge Heraeus Biofuge Strate (Heraeus, Madrid, Spain) were used.

### 2.2. Chemicals and standard solutions

All reagents were of analytical grade. Human serum albumin fraction V (HSA) and human sera were purchased from Sigma (St. Louis, MO, USA); sodium dihydrogen phosphate dihydrate from Fluka (Buchs, Switzerland); Tris-(hydroxymethyl)-aminomethane (Tris) was from Scharlab (Barcelona, Spain), carboxymethylated-β-cyclodextrin was from CycloLab (Budapest, Hungary). Racemic ZPC was kindly donated by Aventis Pharma (Madrid, Spain). Ultra Clear TWF UV deionized water (SG Water, Barsbüttel, Germany) was used to prepare solutions.

Separation buffer in EKC containing Tris 50 mM at pH 6.0 was obtained by dissolving the appropriate amount of Tris in water and adjusting the pH with 1 M HCl. For the incubation process (ZPC–HSA binding) phosphate buffer 67 mM of pH 7.4 was prepared by dissolving the appropriate amount of sodium dihydrogen phosphate dihydrate in water and adjusting the pH with 1 M NaOH. Stock solution of 100 mM CM-β-CD was prepared in the separation buffer. 1000 μM ZPC and HSA stock solutions were daily prepared by weighting the corresponding amount of protein powder and dissolving it with the phosphate buffer.

### 2.3. Methodology

#### 2.3.1. Capillary conditioning

New capillaries were conditioned for 15 min flush with 1 M NaOH at 60 °C [29,31]. This treatment removes adsorbates and refreshes the surface by deprotonation of the silanol groups. Then, they were rinsed for 5 min with deionized water and 10 min with separation buffer at 25 °C. In order to obtain good peak shapes and repeatable migration data, the capillary was conditioned prior to each injection. In all cases, the conditioning run included the following steps: (i) 1 min rinse with deionized water, (ii) 2 min rinse with 0.1 M NaOH, and (iii) 1 min rinse with deionized water at 20 psi. Before chiral selector and sample injection the capillary was also rinsed with the separation buffer for 2 min at 20 psi [31]. No drift in migration time and no peak broadening during a working session (suggesting that no protein adsorption occurred) were verified, indicating the adequacy of the protocol.

#### 2.3.2. Procedure for the enantioseparation of ZPC by EKC using CM-β-CD

For all experiments, the Tris solution was used as electrophoretic buffer. A 30 mM CM-β-CD solution obtained by diluting the stock solution with the buffer was used. ZPC samples (ultrafiltrated fractions and standards) were injected hydrodynamically at 0.5 psi for 5 s. Before sample injection, the capillary was partially filled with the 30 mM CM-β-CD solution by applying 0.5 psi for 99 s. Separation was performed in normal polarity by applying 15 kV (higher voltage would give higher efficiency but also lower enantioresolution [31]). The capillary was thermostated at 25 °C and the UV-detection wavelength was set at 220 nm. The R and S enantiomers were identified by comparison between our *ES* data and the derived from [9] (see Section 3.3.3).

#### 2.3.3. Procedure for the separation and analysis of unbound ZPC fraction to HSA and plasma proteins

We have planned an experimental design to study the binding of the enantiomers to HSA, keeping the concentration of protein nearly physiological values and varying the concentration of ZPC according to the detector capabilities. 5 concentration levels of ZPC were used with 3 independent replicates per level, totalling 15 independent mixtures for the studies with HSA. For the evaluation of the total plasma protein binding, 4 concentration levels of ZPC, with 3 independent replicates per level, totalling 12 samples, were used.

Mixtures containing different ZPC concentrations were prepared in triplicate by the dilution of the stock solutions of drug and protein with the phosphate buffer. HSA concentration was fixed at 475 μM, and in the plasma samples the relation aqueous/plasma solution was 100/300 (v/v). All these mixtures were allowed to reach equilibrium for 30 min in a water bath at 36.5 °C and were