Journal of Chromatography A, 1218 (2011) 3111–3117

Contents lists available at ScienceDirect

Journal of Chromatography A

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

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

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ARTICLE INFO

Article history:
Received 17 January 2011
Received in revised form 7 March 2011
Accepted 14 March 2011
Available online 21 March 2011

Keywords:
Zopiclone
EKC
Human serum albumin
Plasma proteins
Enantioselective binding

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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doi:10.1016/j.chroma.2011.03.025

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

filtered through cellulose filters by centrifugation at 9000 rpm for 30 min. The ultrafiltrate was directly injected into the EKC system.

2.4. Software and calculations

From the experimental data, pseudoeffective (due to the partial filling technique) electrophoretic mobilities corrected for electroosmotic flow (EOF) variations were calculated according to the following equation:

$$\mu_i = \frac{lL}{V}\left(\frac{1}{t_i} - \frac{1}{t_0}\right) \quad (1)$$

where L and l are the total capillary length and the length from the inlet to the detector, respectively, V the run voltage, t_i is the analyte migration time and t_0 is the migration time of methanol, used as non-interacting electroosmotic flow marker. Methanol is detected at 220 nm as a perturbation in the baseline due to a change in the refractive index. Enantiomeric resolution (Rs) was calculated according to:

$$Rs = \frac{1.18(t_2 - t_1)}{w_1 + w_2} \quad (2)$$

where t_1 and t_2 are the migration time of each enantiomer and w_1 and w_2 represent the peak widths at 50% peak heights.

For the ZPC–HSA binding study routines made/adapted in MATLAB® 4.2 were used for calculations. The results generated were verified using EXCEL® and STATGRAPHICS®. In some calculations a SIMPLEX algorithm routine was used (non-linear fitting). We have used two simplified approaches from the general protein-binding model considering m classes of independent active sites on HSA, each with n_i binding sites:

$$r = \frac{b}{P} = \frac{D-d}{P} = \sum_{i=1}^{m} n_i \frac{K_i d}{1 + K_i d} \quad (3)$$

In this equation, r is the fraction of bound enantiomer per molecule of protein, b is the bound concentration of enantiomer and P is the total protein concentration. D and d are the total and free enantiomer concentrations, respectively. All the concentrations must be expressed in molar units (M). For the site i, n_i represents the apparent site-stoichiometry. Eq. (3) assumes negligible non-specific and non-cooperative binding. Two simplified approaches were used here. The first simplified approach assumes $m = 1$ and $n_1 = 1$. Both assumptions should be reasonably valid for low D/P ratios (usual under in vivo conditions) [31], providing:

$$K_1 = \frac{1}{d}\frac{r}{1-r} \quad (4)$$

Eq. (4) is a 1-parameter model, allowing K_1 estimation for each individual D–P–d data and then serving as a source for outliers' identification [31]. To verify the previous approach, an alternative equation, assuming simply $m = 1$ was used, providing:

$$d = \frac{-(1 - K_1 D + n_1 K_1 P) + \sqrt{(1 - K_1 D + n_1 K_1 P)^2 + 4K_1 D}}{2K_1} \quad (5)$$

Eq. (5) is a d-isolated (dependent variable) non-linear form of Eq. (3) when $m = 1$. SIMPLEX optimization was used for estimating the parameters n_1 and K_1, allowing integer n_1 values [31].

3. Results and discussion

3.1. Enantioseparation of ZPC by EKC

Enantioseparation of ZPC in CE has been reported using neutral cyclodextrins in the separation buffer [19,20,22–24]. Even though charged CDs were used initially for the enantioseparation of neutral compounds they also have shown high enantioselectivity towards many basic and acidic compounds. It has been reported that maximum enantioselectivities are obtained when analytes and CDs have opposite mobilities [32–34], due to the counter-current mobility of the chiral selector and the selectand in the separation capillary [8]. In this paper the use of the anionic carboxymethylated-β-cyclodextrin (CM-β-CD) as chiral selector in EKC using the partial filling technique was evaluated accounting to the positive charge of ZPC in a wide pH range (pK_a = 6.9 [35]). The selection of the pH relied on a compromise between of EOF, chiral selector concentration and mobility of ZPC enantiomers, depending on the running buffer pH and driven by their respective pK_a. Initial experiments were carried out with different buffers (citrate, phosphate, Tris and 2-(N-morpholino)ethanesulfonic acid, MES) at pH values between 4 and 8. Among the buffers, Tris at pH 6 provided the best results in terms of resolution, signal-to-noise ratio and analysis time. This solution (50 mM) was selected as running buffer for the optimization process. In these conditions, cationic ZPC migrates through the selector plug allowing the interaction with the anionic CM-β-CD. Detection wavelength was fixed at 220 nm. Lower wavelengths (e.g. 200 and 210 nm were also tested) gave worse signal-to-noise ratio, probably due to the higher contribution of the absorbance of the selector. In these conditions, the initial experiments suggested that sufficient enantioresolution and relatively short analysis time could be achieved; thus we decided to simplify the study employing univariate optimization. The effect of CD concentration, selector plug length, and temperature on the enantioresolution was studied. Fig. 1 shows the effect of the concentration of CM-β-CD. Migration times of enantiomers increase as the CD concentration increases. The observed pseudoeffective electrophoretic mobilities are a combination of electrophoretic mobility of both free and complexed enantiomer. An increase of CD concentration in the capillary provides higher proportion of anionic complexes, increasing their negative electrophoretical mobility and shifting the peaks to longer migration times. As can be seen, complete separation was obtained using a 20 mM CM-β-CD concentration. An improvement in enan-

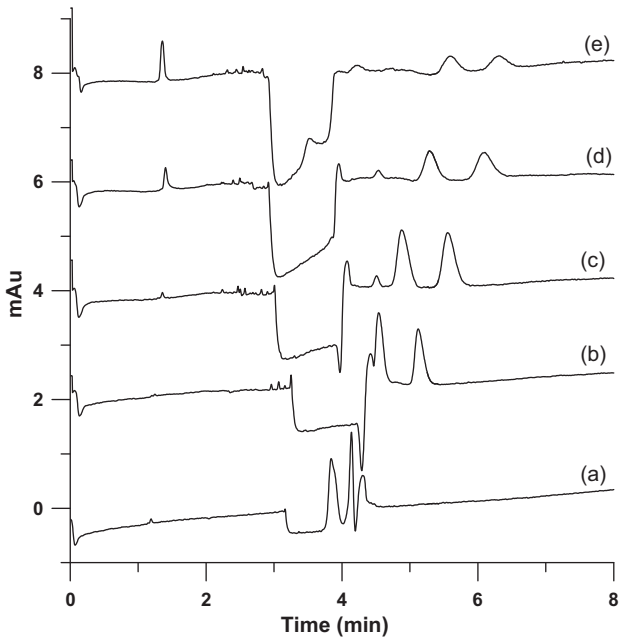

Fig. 1. Effect of the CM-β-CD concentration on the chiral separation of ZPC enantiomers. Electropherograms correspond to CM-β-CD at different concentration levels: (a) 10 mM, (b) 20 mM, (c) 30 mM, (d) 40 mM and (e) 50 mM. Other experimental conditions: ZPC 500 μM, injected at 0.5 psi during 3 s, CD injection 0.5 psi/99 s, 50 mM Tris buffer at pH 6, 25 °C, 15 kV.

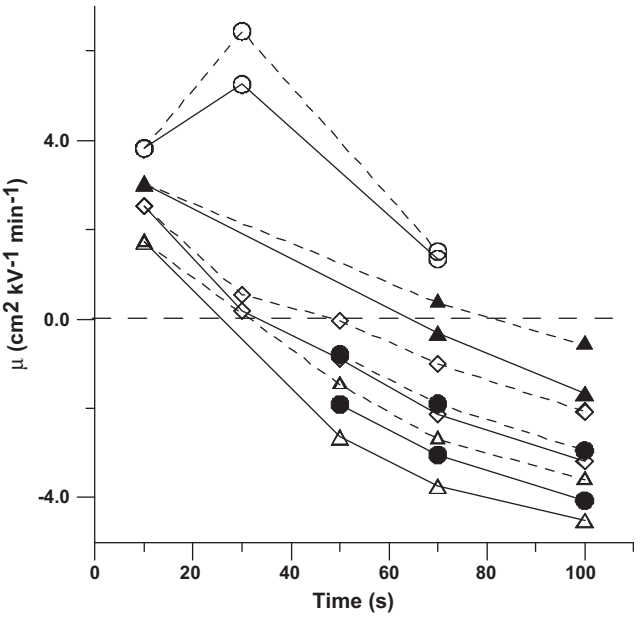

Fig. 2. Effect of the selector plug length on the pseudoeffective electrophoretic mobility S-ZPC (- - -) and R-ZPC (—) at different CM-β-CD concentrations: (○) 10 mM; (▲) 20 mM; (◊) 30 mM; (●) 40 mM; and (△) 50 mM. The solution of CM-β-CD was injected into the capillary by applying 0.5 psi for variable times. Other experimental conditions as in Fig. 1.

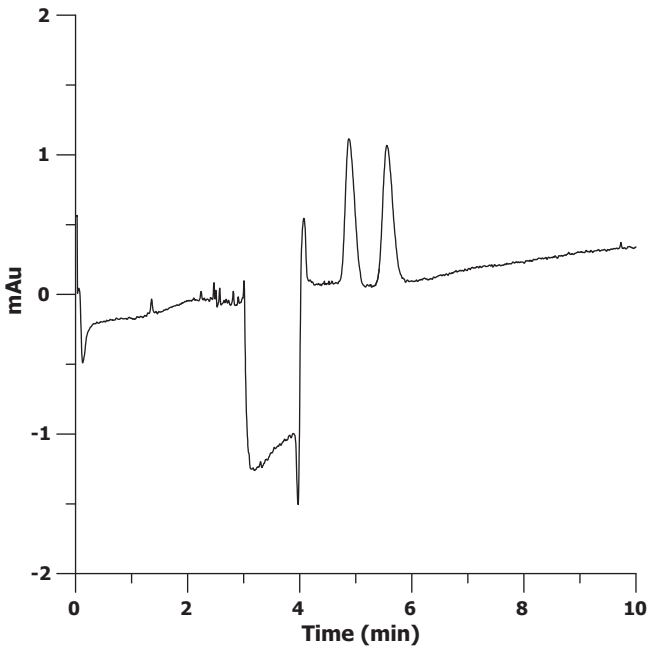

Fig. 3. Electropherogram of racemic ZPC (500 μM) obtained under experimental conditions selected: fused silica capillary (50 μm inner diameter, 31.5 cm total length, 21 cm effective length); Tris buffer (50 mM, pH 6); CM-β-CD 30 mM (injection 0.5 psi/99 s); capillary temperature 25 °C; separation voltage, 15 kV; absorbance detection at 220 nm.

tioresolution upon increasing CM-β-CD concentration is observed, although the shape of the peaks also becomes poorer; probably due to the counter current mobilities of the enantiomers and the chiral selector and the fact that higher CD concentrations provide higher interaction degree (i.e. wider peaks).

When using the partial filling technique, one of the most influential parameters affecting the chiral separation is the amount (the selector plug length, SPL, and concentration) of chiral selector introduced into the capillary. To study this effect on the zopiclone enantioresolution the capillary was filled with increasing concentrations of CM-β-CD by applying 0.5 psi for 10, 30, 50, 70 or 99 s. Fig. 2 shows the SPL effect on the pseudoeffective electrophoretic mobility of both enantiomers, at different concentrations of cyclodextrin. A decrease in pseudoeffective electrophoretic mobility of each enantiomer (or an increase in negative mobility) and an improvement in enantioresolution are observed upon increasing the amount of cyclodextrin introduced into the capillary for all the concentrations assayed. Resolution applying 99 s was sufficiently adequate and higher SPL should provide an increase in the analysis time.

Temperature is considered a key parameter in the chiral separation because it can affect the peaks efficiency, the buffer viscosity and the compound-selector interaction, and consequently can modify the chiral resolution. In this work, temperatures of 15, 25 and 40 °C were tested filling the capillary with a 30 mM CM-β-CD solution (applying 0.5 psi for 99 s). It was observed a decrease in migration times of enantiomers and EOF upon increasing the temperature, both effects are due to a decrease on the viscosity of the running buffer and also to changes in solute–cyclodextrin binding constant. The reduction of migration times resulted in a decrease in the resolution of the enantiomers with increasing temperature (*Rs* values of 2.8, 2.4 and 1.7 were obtained for 15, 25 and 40 °C, respectively), although in all cases a complete chiral separation had been achieved.

From the results obtained the experimental conditions indicated in Section 2.3.2 were selected. Fig. 3 shows the electropherogram obtained with these conditions. As can be seen, a good resolution was obtained for the ZPC enantiomers (*Rs* = 2.1) in an analysis time lower than 6 min.

3.2. Calibration

Calibration standard solutions containing racemic ZPC in the range 50–500 μM were prepared in triplicate. Table 1 shows the regression statistics for the linear regression models, fitted using the least squares approach, for each enantiomer, using the peak area corrected by migration time (in order to compensate for velocity discrepancy between peaks) as dependent variable. It includes the intercept and slope together with their corresponding confidence intervals. These models were used to estimate *d* by interpolating the signals (peak area corrected by migration time) corresponding to ultrafiltrates from the ZPC–HSA mixtures.

3.3. Enantioselective binding to HSA and plasma proteins

Mixtures of racemic ZPC and HSA were designed keeping $D/P < 1$ to assure, as far as possible, model validity [31]. The minimum *D* (~100 μM) was fixed considering the approximate quantification limit for *d*, for both S- and R-enantiomers, d_S and d_R, respectively. *P* was fixed close the physiological concentration (in the hypoalbuminemia-normal levels frontier [36]). Table 2 shows the prepared concentrations (*D* and *P*, in μM units, although they were transformed in M units for modelling), as well as the measured ones (d_S and d_R). The *D*/*P* ratios (from 0.22 to 0.44) should guarantee the

Table 1
Calibration model statistics[a] for S- and R-ZPC under the selected separation conditions using peak area corrected by migration time as dependent variable.

	$b_0 \pm ts$	$b_1 \pm ts$	*r*
S-ZPC	60 ± 40	2.4 ± 0.2	0.993
R-ZPC	30 ± 20	2.37 ± 0.12	0.998

[a] b_0, intercept; b_1, slope; *ts*, confidence interval at the 95% probability level; *r*, correlation coefficient.

Table 2
Experimental design and results. Experimental P was 449.1 μM. Data were transformed to M units for calculations. A robust z-score approach (applied to the corresponding independent K_1 estimates) was used to detect/eliminate outliers (see text for details).

D-level	ID	D (μM)	d_S (μM)	d_R (μM)
1	1	100.8	45.0	52.6
	2	100.8	[b]	82.1
	3	100.8	50.3	63.2
2	4	126.0	[a]	89.7
	5	126.0	[a]	[a]
	6	126.0	73.8	89.3
3	7	151.2	76.7	104.9
	8	151.2	80.8	116.7
	9	151.2	96.5	97.7
4	10	176.4	101.4	127.6
	11	176.4	100.2	130.6
	12	176.4	91.1	111.2
5	13	201.6	97.7	115.4
	14	201.6	118.7	117.9
	15	201.6	91.5	103.6

[a] Data eliminated in the first round.
[b] Data eliminated in the second round.

assumptions made in Section 2.4; for instance, the $m = 1$-model for the ZPC–HSA interaction, since only at high concentration, a single drug may populate multiple sites ($m > 1$) on HSA [31,37]. In Table 2, since each of the five D-concentration levels has three independent replicates, we can control atypical results.

3.3.1. Outlier identification/elimination in the HSA study

The z-score approach has been proposed as an alternative to other tests for outliers, as an automatic criterion [38]. Most tests for outliers depend on good estimates of dispersion, and become less reliable with real errors present. Robust statistics is a convenient method of handling results when they are expected to follow a near-normal distribution, but are suspected to contain a small proportion of errors which are not representative of the bulk of the data [39]. To gain in security, the *median* and *MADe* (a robust estimation of the standard deviation) were used to estimate a robust z-score, z_i for each independent $\log K_{1i}$ estimate, from Eq. (4) [31]:

$$z_i = \frac{\log K_{1i} - median}{MADe} \quad (7)$$

Habitually, $z_i > 3$ are considered unsatisfactory, while $z_i > 2$ are considered doubtable values. Here we have pre-fixed a $z_i > 2.5$ criterion to eliminate a point in a round (see Table 2). The final *median* and *MADe* are included in Table 3. It should be taken into account that irrespectively $n_1 = 1$ or not, this approach could be used to identify and eliminate outliers [31].

3.3.2. Affinity constants to HSA

In Section 3.3.1, Eq. (4) was only used for evaluating outliers assuming that $n_1 = 1$ (the most probable situation at low D/P ratio [31]), since the relatively high concentration of available binding sites. Table 3 provides a robust estimate of $\log K_1$ for the enantiomers based on Eq. (4). To verify the parameters n_1 and K_1 an approach based on non-linear fitting (using Eq. (5)) was used. This is an alternative to other more popular linear approaches having recognised mathematical inconsistencies and risks ([31,40,41] and references therein). SIMPLEX search was started with $n_1 = 1$, $\log K_1 = 4$ (based on results on Table 3), and only integer n_1 values were allowed. The $\log K_1$ estimates found were: 3.37 and 3.14 for S- and R-ZPC, respectively, which agree with values in Table 3. In addition, $n_1 = 1$ estimates were found for both enantiomers, verifying the assumption. As an example of the model adequacy, Fig. 4 shows some results associated to S-ZPC based on Eq. (5); the validation plot for d (Fig. 4a) and the d vs. D plot (Fig. 4b) superimposing on it the fitted model (the best SIMPLEX solution). This suggests that $\log K_1$ estimates in Table 3, including uncertainty (based on a minimum of 12 independent estimates) could be considered acceptable values.

Table 3
Affinity constants of enantiomers of ZPC, protein-binding and enantioselectivity to HSA.

Enantiomer	$\log K_1$ [a]	PB [b]	ES [c]
S-ZPC	3.38 ± 0.14	47 ± 6	1.95
R-ZPC	3.09 ± 0.19	36 ± 8	

[a] From data estimated from Eq. (4) (*median* ± *MADe*; after outliers elimination).
[b] Estimated at $D = 0.2$ μM (total enantiomer concentration) and $P = 600$ μM.
[c] From data estimated from the ratio between $K_{1,S\text{-ZPC}}$ (~2400 M^{-1}) and $K_{1,R\text{-ZPC}}$ (~1230 M^{-1}).

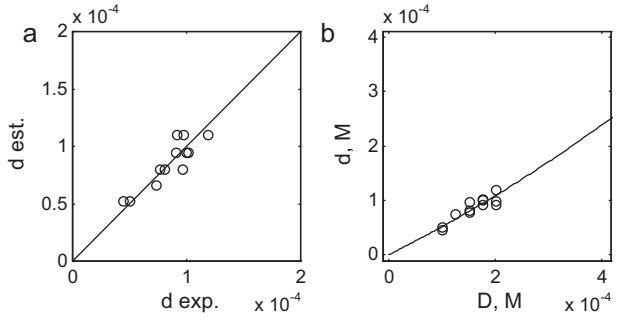

Fig. 4. Results corresponding to S-ZPC using all the data. (a) Validation plot for d (estimated vs. experimental) and (b) d vs. D plot and model (superimposed line; from Eq. (5), optimized by SIMPLEX).

3.3.3. Enantioselectivity and protein binding to HSA

We combined K_1 estimates for S- and R-ZPC to calculate the enantioselectivity of ZPC–HSA interaction ($ES = K_{1,S\text{-ZPC}}/K_{1,R\text{-ZPC}}$) which indicate a fair enantioselectivity degree ($ES \sim 2$), in favour to the first eluted enantiomer, S-ZPC (see Table 3). These results can be compared with the only previous available values, obtained using equilibrium dialysis at 4 °C [9], from which a quantitative estimation of enantioselectivity, not reported by the authors, can be derived ($ES = 524/293 = 1.8$). This value is similar to that obtained in the present work, supporting the fact that ZPC exhibit enantioselective binding to HSA. Attending to this agreement, enantiomers R (the second eluted enantiomer) and S (the first eluted one) were assigned. On the other hand, the reported affinity constants in [9] are notably lower that those encountered here (by a factor close to 4.4). This could be explained in terms of experimental conditions, mainly the incubation temperature.

A key parameter from a pharmacokinetic point of view is the estimation of the percentage of protein binding. By means of Eq. (5) and using the constants in Table 3 at prefixed (desired) D and P values, it is possible to estimate the corresponding d values, and from them, the PB according to:

$$PB = \frac{100(D - d)}{D} \quad (8)$$

Here we have assumed values consistent with physiological conditions, e.g. $D = 0.2 \times 10^{-6}$ M (see [42]) and $P = 600 \times 10^{-6}$ M (see Section 3.3). After d_S and d_R estimation, PB for each enantiomer was calculated (see Table 3). As expected (according to the K_1 values), binding percentages to HSA reported in [9], 23.9 ± 2.8 and 15.0 ± 3.5 for S- and R-ZPC, respectively, are lower than those found here (Table 3), but also too low (in our opinion) compared with those encountered for plasma by the authors.

Table 4
Plasma protein binding values for racemic ZPC and its enantiomers. Some available aspects that could potentially explain differences between results were included in footnotes.

PB% rac-ZPC	*PB*% S-ZPC	*PB*% R-ZPC	Ref.
45[a]	–	–	Gaillot et al. [25]
78.7–91.2[a,b]	–	–	Howard et al. [26]
79.3 ± 5.5[b,c]	75.1 ± 2.1[b,c]	86.8 ± 5.2[b,c]	Fernandez et al. [9]
45[d]	–	–	AEMPS [27]DrugBank [28]
–	52–59[d]	–	DrugBank [28]
47 ± 4	45 ± 3	49 ± 6	This work

[a] In vivo (presumably $D/P \ll 1$).
[b] Equilibrium dialysis was used to separate free and bound ZPC fractions.
[c] In vitro; plasma with EDTA or heparin as anticoagulant; incubation at 4 °C (24 h); $D/P = 0.00043$. The authors also reported other in vitro results but using citrate-phosphate-dextrose as alternative anticoagulant (38.0 ± 8.1, 31.8 ± 6.6, 42.5 ± 7.1, respectively) and in vivo estimates for R- and S-ZPC (86.76 and 79.17, respectively; presumably $D/P \ll 1$).
[d] No references are provided (it is assumed the values comes from in vivo experiments; presumably $D/P \ll 1$).

3.3.4. Plasma protein binding

The total plasma protein binding was approximated for racemic ZPC and its enantiomers, using Eq. (8) according with our experimental data using human sera. These data were compared with previous values found in the literature (Table 4), illustrating that this was an unresolved matter. Our *PB* result on racemic ZPC is consistent with some reported ones [25,27,28], but differs from others [9,26]. In addition, our result for S-ZPC is reasonably close with the one reported from the pure enantiomer [3]. Comparing our results for plasma (Table 4) with those for HSA (Table 3), it seems that S-ZPC binds mainly to HSA, while R-ZPC binds also to other plasma proteins, but with a limited affinity compared to HSA. This also disagrees with the explanation provided in [9] forced by the large *PB* differences between HSA and AGP and plasma.

3.3.5. Final remarks

The protocol used, including (i) elimination of outliers (Eq. (4) + Eq. (7)) on K_1 direct individual estimations and (ii) verification of $n_1 = 1$ and K_1 (Eq. (5)) aims to provide a sufficient quality on the results. To gain in robustness we have avoided unfavourable D/P ratios (to guaranty the validity of the assumed model along the *D*-concentration interval), then using a short-*D*-concentration interval [31]. The estimations from Eq. (4) increase the control over individual data (here replicates at different *D*-concentration levels) allowing the application of univariate statistics instead off a regression model with a single estimation. It should be noted that using Eq. (4) (independent estimations were obtained) the *D*-range becomes irrelevant, i.e. the estimation is not based on regression where a short range would become problematic.

D, *P* or D/P information, as well as the experimental *d*-values and experimental details should be always clearly reported since it could condition the reported estimates (allowing appropriate comparison or even a different mathematical data treatment).

4. Conclusions

Due to its intrinsic characteristics such as high separation efficiencies, speed of analysis, low reagent consumption and small sample requirements, EKC, combined here with CM-β-CD using the partial filling technique, has been demonstrated to be adequate for the inexpensive chiral separation of ZPC enantiomers, allowing the evaluation of enantioselective binding of ZPC to HSA and total plasma proteins. The results suggest that S-ZPC exhibit more affinity (by a factor of 2) to HSA than R-ZPC. This work provides the second evidence of ZPC enantioselectivity to HSA, using an independent method with more favourable experimental conditions respect to the physiological ones, and therefore serves to confirm the previous result. Our *PB* results (47 ± 4, 49 ± 6 and 45 ± 3% for racemic, R- and S-ZPC, respectively) agree with other reported values, supporting the idea that the protein binding of ZPC is in the ∼45% level (e.g. the value that figures in the DrugBank database [28]) and not in the other reported level (∼80%).

When performing protein binding studies (enantioselective, as in this case, or not) involving HSA, the mathematical approaches to be selected should be consistent to the selected experimental design. Factors to be considered include total enantiomer (or drug) and protein, *D* and *P*, concentration intervals used, which also affect the interval of other variables used, e.g. *d* or *r* data, and D/P ratios. When possible, model assumptions should be a priori justified, but also verified. The approaches selected have consequences related to both accuracy and uncertainty of the estimates. Finally, data quality indicators should be considered (e.g. outliers) and robust statistics are recommended.

Acknowledgments

The authors acknowledge the Spanish Ministry of Science and Innovation (MCINN), the European Regional Development Fund (ERDF) (project SAF2008-00859) for the financial support. Dr. Salvador Sagrado Vives acknowledges the South African–Spanish Bilateral Agreement of the National Research Foundation (NRF), South Africa, project UID 69160 and the Spanish Ministry of Science and Innovation (MCINN), project HS2008-0002 for the financial support. The authors declared no conflict of interest.

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