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Evaluation of enantioselective binding of propanocaine to human serum albumin by ultrafiltration and electrokinetic chromatography under intermediate precision conditions

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ABSTRACT

Stereoselectivity in protein binding can have a significant effect on the pharmacokinetic and pharmacodynamic properties of chiral drugs. In this paper, the enantioselective binding of propanocaine (PRO) enantiomers to human serum albumin (HSA), the most relevant plasmatic protein in view of stereoselectivity, has been evaluated by incubation and ultrafiltration of racemic PRO–HSA mixtures and chiral analysis of the bound and unbound fractions by electrokinetic chromatography using HSA as chiral selector. Experimental conditions for the separation of PRO enantiomers using HSA as chiral selector and electrokinetic chromatography have been optimised. Affinity constants and protein binding in percentage (PB) were obtained for both enantiomers of PRO, as well as the enantioselectivity (ES) to HSA. Data were obtained in two independent working sessions (days). The influence of the session and fraction processed factors were examined. A univariate direct-estimation approach was used facilitating outliers' identification and statistical comparison. Non-linear fitting of data was used to verify the stoichiometry and affinity estimations obtained by the direct approach. Robust statistics were applied to obtain reliable estimations of uncertainty, accounting for the factors (day and processed fraction), thus representing intermediate precision conditions. Mimicking *in vivo* experimental conditions, information unapproachable by *in vivo* experiments was obtained for PRO enantiomers interacting with HSA. For the first (E1) and the second (E2) eluted PRO enantiomers the results were: 1:1 stoichiometry, medium affinity constants, $\log K_{E1} = 3.20 \pm 0.16$ and $\log K_{E2} = 3.40 \pm 0.14$, medium protein binding percentage, PB = 48.7 and 60.1% for E1 and E2, respectively, and moderate but significant enantioselectivity, $ES = K_{E2}/K_{E1} = 1.5 \pm 0.3$.

1. Introduction

Many drugs are non-covalently bound to serum proteins or other binding agents in circulation, a feature that makes the determination of free drug fractions and studies of drug-binding to serum proteins a topic of great interest in clinical and pharmaceutical research [1]. For racemic drugs, very often one of the enantiomers is the most active while the other may produce side-effects and even toxicity in some cases. Pharmacological differentiation of enantiomers may occur at pharmacodynamic and pharmacokinetic levels. In view of the distribution process through the body, the differences between the enantiomers behaviour may be due mainly to the differences in their binding to the different plasma proteins [2]; among them, human serum albumin (HSA), the most abundant protein in the circulatory system (i.e. with the largest complexation potential), exhibits the highest potential enantioselectivity, becoming a key piece for pharmacokinetically characterizing chiral xenobiotics. Methodologies able to obtain protein binding information play an important role in the development of new drugs and new formulations as well as in investigations on bioavailability and metabolism. Furthermore, they have often become the basis of therapeutic drug monitoring and drug management in patients [3].

A review of our group [4] emphasised the microseparation techniques for evaluating the enantioselective binding of drugs to plasma proteins. It includes several reports using a methodology that allowed us to perform the study from the racemate of the target molecule. The methodology is conducted in several steps: (i) equilibration (incubation) of racemic xenobiotic and protein mixtures according to a given experimental design; (ii) separation of the free xenobiotic (unbound fraction) and xenobiotic–protein complex (bound fraction) by ultrafiltration; (iii) determination of the

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enantiomer concentrations in one of the fractions (free or bound) by means of capillary electrophoresis using a chiral selector (e.g. HSA); and (iv) parameter estimation (e.g. protein binding (percentage), PB, number of binding sites or stoichiometry, affinity constants and enantioselectivity to the protein) following a given mathematical strategy.

While the separation/analytical aspects have been extensively investigated, the impact of the experimental design (in the incubation step) and the mathematical approach (involving several assumptions and model simplification) on the results has been underestimated in the past, entailing a risk of lack of quality results [5,6], or an underestimation of the process uncertainty [6]. Regarding the uncertainty, studies under intermediate precision conditions (e.g. different working sessions or procedures for free and bound fractions) to check the impact on the affinity estimates have not been found in the literature.

Enantiomers of local anaesthetics have been claimed to exhibit large differences in their pharmacological/toxicological behaviour; however, up to now, there is not information for propanocaine (PRO). For the first time, the evaluation of the enantioselective binding of PRO to HSA is carried out in this work. Chiral separation of PRO enantiomers is optimised using HSA as chiral selector and electrokinetic chromatography (EKC)-partial filling technique. Affinity constants of enantiomers are obtained using an experimental design which accounts for intermediate precision conditions (accounting for between-day and between processed fraction variability) and a recently proposed univariate direct-estimation approach [6]. This approach allows outliers identification/elimination, decision based on hypothesis testing and uncertainty estimation. The effects of working session and procedure related to the free/bound fraction factors on the estimates are studied. The enantioselectivity of HSA for PRO enantiomers as well as the protein binding percentage at approximate physiological conditions are also estimated. The results obtained in this paper, based on ultrafiltration and electrokinetic chromatography, using HSA as chiral selector, provide the first affinity and *PB* estimates for PRO enantiomers and represent the first evidence of the enantioselective binding of propanocaine to HSA.

2. Experimental

2.1. Instrumentation

A Hewlett-Packard HP ^{3D}CE capillary electrophoresis system (Agilent Phoenix, AZ, USA) equipped with a diode array detector (DAD) and HP ^{3D}CE Chemstation software was used. Fused-silica capillaries (Polymicro Technologies, Phoneix, AZ, USA) of 50 μm inner diameter (i.d) and 363 μm outer diameter (o.d) with total and effective lengths of 56 and 47.5 cm, respectively, were used. 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 the buffer solutions.

A thermostatised bath (JP Selecta, Barcelona, Spain) was used for sample incubation. For the ultrafiltration of samples, Amicon Ultra 0.5 ml centrifugal filters of a molecular weight cut-off 10,000 Da MWCO (Millipore Corporation, Bedford, MA, USA) and a centrifuge (Orto Alresa, Alvarez-Redondo, S.A., Madrid, Spain) were used.

2.2. Chemicals and standard solutions

All reagents were of analytical grade. Human serum albumin fraction V (HSA) was purchased from Sigma (St. Louis, MO, USA); sodium dihydrogen phosphate dihydrate from Fluka (Buchs, Switzerland); racemic propanocaine (PRO) was kindly donated by Laboratorios Seid, S.A. (Lliça de Vall, Barcelona, Spain). Tris–(hydroxymethyl)-aminomethane (Tris) was from Scharlab (Barcelona, Spain). Ultra Clear TWF UV deionised water (SG Water, Barsbüttel, Germany) was used to prepare solutions.

Separation buffer in EKC containing 50 mM Tris at pH 8.0 was obtained by dissolving the appropriate amount of Tris in water and adjusting the pH with 1 M HCl. 67 mM phosphate buffer at pH 7.4 was prepared for the study of binding to HSA by dissolving the appropriate amount of sodium dihydrogen phosphate dihydrate in water and adjusting the pH with 1 M NaOH. 1600 μM HSA stock solution was daily prepared by weighting the corresponding amount of protein powder and dissolving it with 67 mM phosphate buffer.

A stock standard solution of racemic PRO 1000 μM was prepared in phosphate buffer. In order to obtain the calibration graphs, working solutions of PRO containing different concentrations of enantiomers (between approx. 150 and 400 μM of each enantiomer) were prepared in duplicate by diluting the stock standard solution with phosphate buffer.

2.3. Methodology

2.3.1. Procedure for racemic PRO–HSA mixture incubation and separation of free/bound PRO fractions

Mixtures containing different racemic PRO and HSA concentrations (500 μL) were prepared by dilution of the stock solutions of drug and protein with 67 mM phosphate buffer at pH 7.4. All these mixtures were allowed to reach equilibrium for 30 min (incubation) in a water bath at 36.5 °C and were filtered through regenerated cellulose filters by centrifugation (ultrafiltration) at 9000 rpm for 30 min. The ultrafiltrate contains the free fraction from each mixture. On the other hand, the residue, that contains the bound fraction, was removed from filters by turning them the right way up and centrifugating at 9000 rpm for 2 min. 200 μL of acetonitrile were added to the residue to provoke the precipitation of HSA and the liberation of the bound drug, and centrifuged at 9000 rpm for 10 min at 25 °C. The supernatant contains the bound fraction from each mixture.

2.3.2. Capillary conditioning

The new capillary was conditioned by flushing with 1 M NaOH for 10 min at 60 °C. Then, it was rinsed for 5 min with water and 15 min with phosphate buffer at 30 °C. In order to obtain good peak shapes and reproducible migration times, the capillary was conditioned at the beginning of the working session and between runs with the following sequence: (i) 2 min rinse with deionised water, (ii) 2 min rinse with 1 M NaOH, (iii) 2 min rinse with deionised water, and (iv) 2 min rinse with running phosphate buffer at 1000 mbar.

2.3.3. Procedure for the enantioseparation of PRO by EKC using HSA as chiral selector

A 50 mM Tris solution of pH 8.0 was used as electrophoretic buffer. A 190 μM HSA solution was daily prepared in electrophoretic buffer. Solutions (calibration standards and free and bound fractions) were injected hydrodynamically at 50 mbar for 3 s. Before injection, the capillary was partially filled with HSA solution by applying 50 mbar pressure for 180 s. The capillary cassette temperature was set at 30 °C and a running voltage of 15 kV was used. UV detection was performed at 220 nm. Under the selected separation conditions, the resolution obtained for the PRO enantiomers was >1.3 and the analysis time lower than 6 min.

2.4. Software and calculations

EXCEL®, STATGRAPHICS® and routines made/adapted in MATLAB® 4.2 were used for calculations. In some calculations a SIMPLEX algorithm routine was used (non-linear fitting). Eq. (1) represents the general protein-binding model considering m classes of independent active sites on HSA, each with n_i binding sites:

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

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 are expressed in molar units (M). For the site i, n_i represents the apparent site-stoichiometry. Eq. (1) assumes negligible non-specific and non-cooperative binding. Two simplified approaches from Eq. (1) were used. The first one assumes $m = 1$ and $n_1 = 1$ [6], providing:

$$K_1 = \frac{1}{d}\frac{r}{1-r} \quad (2)$$

This equation allows direct (no regression, no fitting) K_1 estimation for each individual D–P–d data obtained for a given experimental design. This strategy allows univariate data treatment of K_1 (or $\log K_1$) estimates and therefore, outliers' identification [6]. To verify the previous approach, an alternative equation, assuming simply $m = 1$ was used, providing:

$$d = \frac{-(1-K_1D+n_1K_1P)+\sqrt{(1-K_1D+n_1K_1P)^2+4K_1D}}{2K_1} \quad (3)$$

Eq. (3) is a d-isolated (dependent variable) non-linear form of Eq. (1) when $m = 1$. SIMPLEX optimisation was used for estimating the parameters n_1 and K_1. Both $m = 1$ and $n_1 = 1$ assumptions should be reasonably valid for low D/P ratios, which is the usual situation under *in vivo* conditions [6].

2.5. Experimental design

The independent variables (D and P concentrations) were designed using $D/P < 1$ as a prefixed rule. The different D and P mixtures were generated as described in Section 2.3.1, and the PRO enantiomers were separated as described in Section 2.3.3. The UV signal from the EKC system generated two peaks, E1 and E2 corresponding to the first and second eluted enantiomers, respectively, which along with the calibration models were used to calculate d_{E1} and d_{E2}. From this data using Eq. (2), K_{E1} and K_{E2} were estimated and the subsequent parameters for each D–P–d data set were calculated.

P values were fixed close to the physiological concentration (around the hypoalbuminemia-normal levels frontier [6]). For the highest P value (~475 μM), D values under ~250 μM did not provide adequate analytical signals (corrected peak areas) for enantiomers in order to perform their quantification (d_{E1} and d_{E2}), so a safe minimum D (~265 μM), convenient for quantification purposes, was fixed. The maximum D (~370 μM) was chosen considering the different P values and the $D/P < 1$ rule. Table 1a shows the design of experiments, DOE, of thirteen levels (different D–P concentrations) used. The P values shown in Table 1a correspond to the first day in which the DOE was processed. The DOE was repeated during a second day using a new HSA solution (P values were slightly larger; see Table 1b). Table 1b shows the free enantiomer concentrations, d_{E1} and d_{E2}, obtained from the analysis of the free and bound fractions (F and B, respectively) in the order (run) in which the samples were analyzed in the EKC system after a randomisation process. For three experiments (the free fraction from DOE level 8 corresponding to days 1 and 2 and the free fraction from DOE level 13 corresponding to day 2) d-data were not obtained due to problems with the electrophoretic system (not included in Table 1b).

Table 1a
Design of experiments (DOE) showing the independent variables (D and P used as mixtures for incubation) and the D/P ratio.

DOE level[a]	D (M)	P (M)[b]	D/P
1	0.000265	0.000471	0.56
2	0.000291	0.000377	0.77
3	0.000291	0.000408	0.71
4	0.000291	0.000471	0.62
5	0.000318	0.000345	0.92
6	0.000318	0.000377	0.84
7	0.000318	0.000393	0.81
8	0.000318	0.000408	0.78
9	0.000318	0.000471	0.68
10	0.000344	0.000377	0.91
11	0.000344	0.000408	0.84
12	0.000344	0.000471	0.73
13	0.000371	0.000471	0.79

[a] Concentration levels of the experiments (ordered by D and then P values).
[b] The P values correspond to day 1. The DOE was repeated a second day in which P values were slightly larger (see Table 1b) since a new protein solution was prepared.

3. Results and discussion

3.1. Optimisation of chiral separation of (±)-PRO enantiomers by EKC-partial filling technique using HSA as chiral selector

The chiral separation of compounds using HSA as chiral selector in the EKC-partial filling technique depends on several experimental variables such as protein concentration, running pH (background electrophoretic buffer and protein solutions), chiral selector plug length (SPL), background electrolyte concentration, running temperature and voltage. Studies carried out in our laboratory using this technique pointed out that the critical experimental variables determining enantioresolution are the running pH, HSA concentration and plug length [7,8]. In this paper, several experiments were carried out in order to optimise the experimental conditions for chiral separation of propanocaine (see Fig. 1).

Since pH is the most critical variable that determines enantioresolution with HSA, experiments in the 7–8.5 pH range were made. For this purpose, the following experimental conditions, selected according to previous experiments were fixed: electrophoretic buffer concentration (50 mM Tris), applied voltage (15 kV), temperature (30 °C), HSA concentration (190 μM) and plug length (150 s). As can be observed in Fig. 1a, partial resolution of enantiomers was achieved in the 8.0–8.5 pH range (R_s = 0.99 and 0.73, respectively). At pH 8, the effects of HSA concentration (160–190 μM, Fig. 1b) and the selector plug length (150–180 s, Fig. 1c) on the enantioresolution of propanocaine were also studied. Maximum resolution (R_s = 1.3) was obtained in the following experimental conditions: electrophoretic buffer composed of 50 mM Tris at pH 8; 190 μM HSA solution at pH 8 applied at 50 mbar for 180 s as chiral selector; electrophoretic runs performed at 30 °C applying 15 kV voltage.

3.2. Evaluation of enantioselective binding of (±)-PRO enantiomers to HSA

As stated in the introduction, the methodology was already proposed, optimised and validated by the authors ([4,6,7] and references therein), and the data found in this case exhibit the same pattern found in previously reported results for other drugs. Therefore, only the specific information concerning to PRO is reported here.

Table 1b
Results including the measured variables (d_{E1} and d_{E2}). The order (run; after a randomisation process) in which samples were processed in the EKC system is indicated.

Day	Fraction[a]	Run	DOE level (Table 1a)	D (M)	P (M)	d_{E1} (M)	d_{E2} (M)
1	F	1	6	0.000318	0.000377	0.000217	0.000165
	F	2	11	0.000344	0.000408	0.000223	~~0.000150~~ [b]
	F	3	5	0.000318	0.000345	0.000240	0.000188
	B	4	8	0.000318	0.000408	0.000187	0.000166
	B	5	5	0.000318	0.000345	0.000187	0.000163
	F	6	10	0.000344	0.000377	0.000255	0.000204
	F	7	12	0.000344	0.000471	0.000208	0.000178
	B	8	7	0.000318	0.000393	0.000217	0.000199
	B	9	1	0.000265	0.000471	0.000158	0.000144
	F	10	13	0.000371	0.000471	0.000263	0.000209
	B	11	3	0.000291	0.000408	0.000181	0.000171
	F	12	7	0.000318	0.000393	~~0.000272~~ [b]	0.000175
	B	13	9	0.000318	0.000471	0.000192	0.000186
	B	14	11	0.000344	0.000408	0.000238	0.000222
	F	15	2	0.000291	0.000377	0.000191	0.000155
	F	16	1	0.000265	0.000471	0.000162	0.000124
	F	17	4	0.000291	0.000471	0.000171	0.000137
	B	18	4	0.000291	0.000471	0.000180	0.000164
	B	19	2	0.000291	0.000377	0.000162	0.000142
	B	20	10	0.000344	0.000377	0.000210	0.000192
	F	21	3	0.000291	0.000408	0.000203	0.000150
	B	22	13	0.000371	0.000471	0.000215	0.000191
	B	23	6	0.000318	0.000377	0.000197	0.000181
	B	24	12	0.000344	0.000471	0.000172	0.000160
	F	25	9	0.000318	0.000471	0.000179	0.000157
2	B	26	11	0.000344	0.000414	0.000261	0.000240
	B	27	12	0.000344	0.000478	0.000209	0.000187
	B	28	1	0.000265	0.000478	0.000181	0.000162
	F	29	4	0.000291	0.000478	0.000214	0.000154
	F	30	2	0.000291	0.000382	0.000193	0.000180
	B	31	4	0.000291	0.000478	0.000204	0.000183
	B	32	10	0.000344	0.000382	0.000239	0.000215
	B	33	9	0.000318	0.000478	0.000219	0.000206
	F	34	1	0.000265	0.000478	0.000193	0.000140
	F	35	6	0.000318	0.000382	~~0.000273~~ [b]	0.000217
	F	36	5	0.000318	0.000350	0.000255	0.000206
	B	37	13	0.000371	0.000478	0.000248	0.000218
	B	38	2	0.000291	0.000382	0.000189	0.000164
	B	39	6	0.000318	0.000382	0.000223	0.000186
	F	40	11	0.000344	0.000414	0.000209	~~0.000149~~ [b]
	F	41	3	0.000291	0.000414	0.000225	0.000182
	F	42	12	0.000344	0.000478	0.000255	0.000202
	F	43	10	0.000344	0.000382	~~0.000291~~ [b]	0.000230
	F	44	7	0.000318	0.000398	~~0.000273~~ [b]	~~0.000246~~ [b]
	B	45	3	0.000291	0.000414	0.000205	0.000189
	B	46	7	0.000318	0.000398	0.000239	0.000217
	B	47	8	0.000318	0.000414	0.000215	0.000189
	F	48	9	0.000318	0.000478	0.000225	0.000184
	B	49	5	0.000318	0.000350	0.000215	0.000201

[a] d data were obtained independently after processing the free fraction (F) and the bound fraction (B).
[b] Eliminated after applying the $|z| > 3$ criterion on $\log K_1$ estimates from the D–P–d data using Eq. (2) (see Section 3.1).

Racemic PRO–HSA mixtures were prepared according the experimental design described in Section 2.5. Mixtures were incubated and the free/bound PRO fractions were separated using the procedure described in Section 2.3.1 and analyzed by means of EKC in the optimum enantioseparation conditions described above.

Simultaneously, to estimate the d_{E1} and d_{E2} values for each experimental condition shown in Table 1b, calibration with standards of the PRO racemate were processed along the two-days where samples were assayed.

Six enantiomer concentration levels (159, 212, 265, 318, 371 and 424 μM; d_{E1} and d_{E2}) were used for calibration. For the first day, the linear regression equations were Corrected peak area = $-0.48 + 0.0085\, d_{E1}$ (μM), $R^2 = 0.990$, and Corrected peak area = $-0.33 + 0.0084\, d_{E2}$ (μM), $R^2 = 0.997$. Similar results (not shown) were obtained during the second session. As an example, Fig. 2 shows the result obtained for the analysis of the free fraction corresponding to run 48 in Table 1b (D = 318 μM, P = 478 μM) together with the calibration standard containing 318 μM of each enantiomer. After that, the D–P–d values were introduced in Eq. (2) to estimate the K_1 data set.

3.2.1. Outlier identification/elimination

The experimental protein binding processes is complex; however, there are clearly identifiable sources of uncertainty. For instance, in the case of PRO enantiomers (analytes) from a racemate–HSA incubation process, the uncertainty could be from the preparation of standards and mixtures, incubation, ultrafiltration, analytical separation and measurement, etc. Among them, in this kind of studies, the binding degree during the incubation and the separation of the unbound fraction during ultrafiltration presumably become the most critical factors. Consequently, an impact to d-data imprecision is expected, also concerning the subsequent estimates.

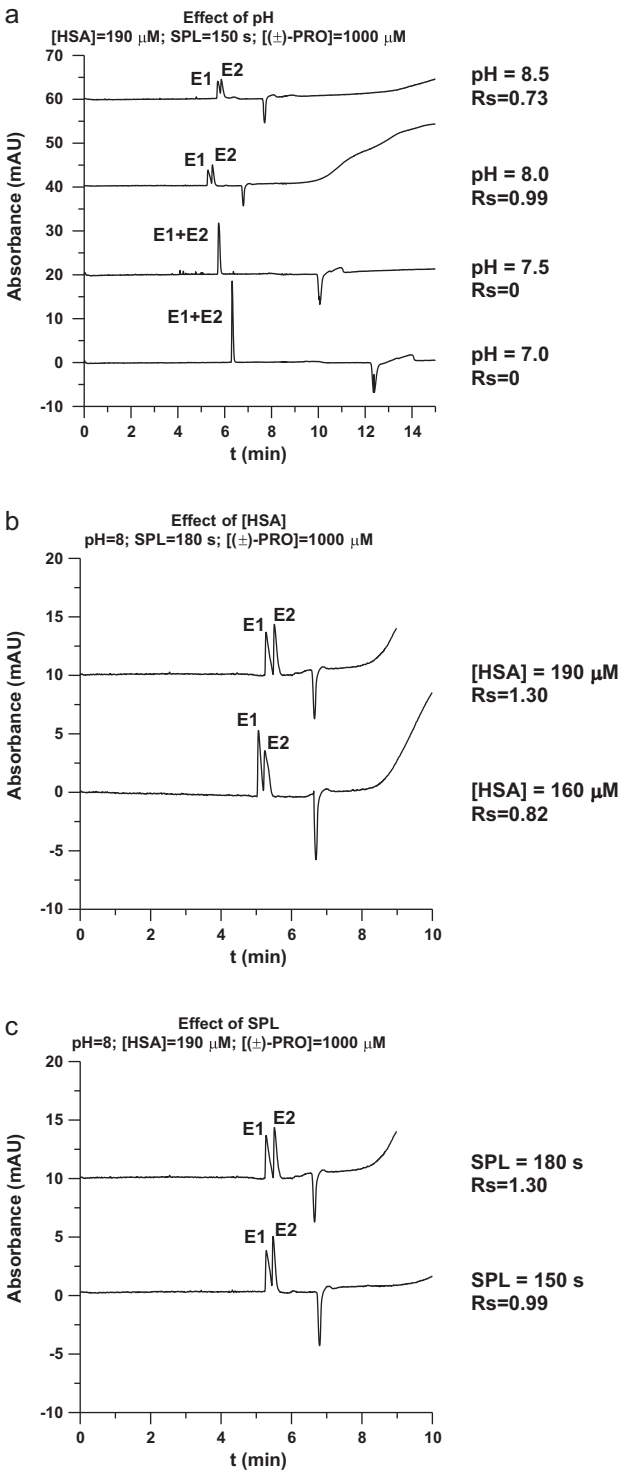

Fig. 1. Optimisation results for separation of (±)-PRO enantiomers. Effects of the (a) running pH, (b) concentration of HSA and (c) selector plug length, SPL. Other experimental conditions: 1000 μM racemic propanocaine standard solution, 50 mM Tris as electrophoretic buffer and electrophoresis carried out at 30 °C and 15 kV. UV detection at 220 nm. First (E1) and second (E2) eluted enantiomer.

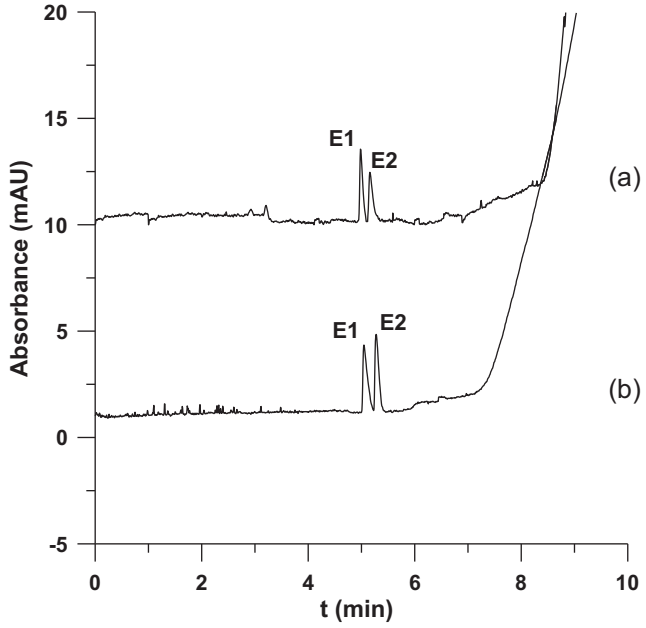

Fig. 2. Electropherograms obtained for the analysis of: (a) the free fraction corresponding to run 48 in Table 1b (D = 318 μM, P = 478 μM) and (b) the calibration standard containing 318 μM of each enantiomer. Electrophoretic conditions: electrophoretic buffer composed of 50 mM Tris at pH 8; 190 μM HSA solution at pH 8 applied at 50 mbar for 180 s as chiral selector; electrophoretic runs performed at 30 °C applying 15 kV voltage. UV detection at 220 nm. First (E1) and second (E2) eluted enantiomer.

Outliers identification is better performed on a univariate basis, therefore, the individual K_1 estimates (and their corresponding log K_1 values) obtained from all the experiments in Table 1b, were used together. The log K_1 data vectors of the enantiomers ($\log K_{E1}$ and $\log K_{E2}$) were submitted to analysis of outliers according to the z-score approach (based on the *mean* and standard deviation, s of each vector). The z-score calculated for each value, $z_i = (\log K_{1i} - \text{mean})/s$, allows to locate those values with $|z| > 3$ (considered unsatisfactory in successive rounds, after eliminating $|z| > 3$ data and recalculating the mean and s). For E1, four $\log K_{E1}$ values, providing $|z| > 3$ (runs 12, 35, 43 and 44) were considered outliers (the corresponding d_{E1} values are marked in Table 1b). For E2, three $\log K_{E2}$ values, providing $z > 3$ (runs 2, 40 and 44) were considered outliers (the corresponding d_{E2} are marked in Table 1b). Note that this analysis is based on mixing the $\log K_1$ data of two days (intermediate precision conditions) and two fractions per DOE level, but is performed independently for each enantiomer, so an outlier found in a run for an enantiomer does not correspond to an outlier for the other.

3.2.2. Study of factors effect on estimates

In Fig. 3 the z-scores values corresponding to $\log K_1$ were grouped and plotted separately considering the day (session) and processed fraction factors for both enantiomers (Fig. 3a and b for E1 and E2, respectively). Those data marked with an asterisk correspond to the outliers found in Section 3.2.1. Fig. 3 allows exploring the influence of these two factors on $\log K_1$ estimates. This study has not previously performed related to affinity constant results, only the processed fraction influence on PB results has been reported [7]. First, it could be observed that for both enantiomers, outliers correspond to the free fraction data (j = 1 and 3). However, after outliers elimination, the remaining free fraction data exhibit a similar dispersion than the bound fraction data (j = 2 and 4), particularly for E2. Globally, it seems that data from the second day (j = 3 and 4) provide slightly lower z-scores (more visible on E2 results). Also, the effect of working with the free or the bound fraction becomes less important than the day-effect (particularly from data obtained during the second session).

After eliminating the outliers, the $\log K_{E1}$ and $\log K_{E2}$ data vectors, together with the information of the factors day and fraction, were submitted to multifactor ANOVA (Type III sums of squares have been chosen, so the contribution of each factor is measured

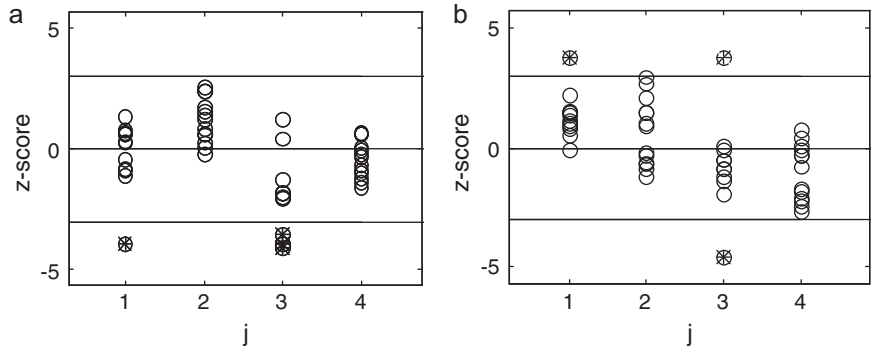

Fig. 3. *z*-Scores (corresponding to log K_1 estimates from Eq. (2); see Section 3.1 for details) grouped by the day and processed fraction factors, plotted for both enantiomers. Outliers (those outside the $z = 3$ lines and marked with an asterisk) were eliminated for further calculations. The index *j* indicates the criteria used for grouping the data (1 = first day – free fraction; 2 = first day – bound fraction; 3 = second day – free fraction; 4 = second day – bound fraction). (a) E1 enantiomer; (b) E2 enantiomer.

having removed the effect of the other). Except for the processed fraction effect in the case of log K_{E2} ($P = 0.15$), the *P*-values were lower than 0.05 indicating a statistically significant effect at the 95.0% confidence level. The inclusion of the interaction between factors does not change these conclusions. However, initially, there is not a reason to suspect that the data from a particular session or fraction procedure is worse than the other conditions, so, after eliminating the outliers, the remaining data in Table 1b were combined to derive the final affinity estimates, i.e. the averaged log K_{E1} and log K_{E2} values as well as the averaged enantioselectivity (ES = K_{E2}/K_{E1}) and their corresponding uncertainties. Therefore, the estimated uncertainty reflects the overall process variability (the data accounts for the previously mentioned sources of uncertainty; Section 3.1) and the fact of working under intermediate precision conditions (different days and fractions processed), an unexplored strategy.

3.2.3. Robust estimates and model assumption discussion/verification

The data vectors (without outliers) representing the enantiomers' affinity (AF, in terms of log K_1) and the enantioselectivity (ES) to HSA, where first explored graphically. Fig. 4 shows the Box-and-Whisker plots for AFE1 and AFE2 (log K_{E1} and log K_{E2}) and ES estimates. As can be seen, AFE1 and AFE2 exhibit similar dispersion with median and mean values almost coincident; while ES (combined K_1 data from E1 and E2) present larger dispersion with an appreciable difference between the mean and median values. Robust statistics are more convenient when data are expected to follow a near-normal distribution, but are suspected to contain a small proportion of errors [8], like the present data (particularly for

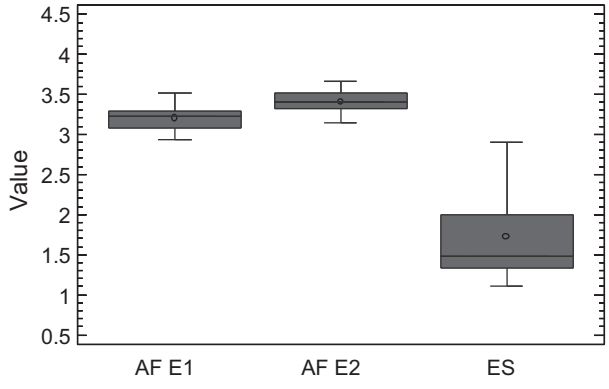

Fig. 4. Box-and-Whisker plot for affinity estimates (log K_1 values) for enantiomers (AF E1 and AF E2) and enantioselectivity (ES, estimated as K_{E2}/K_{E1}). Means (○) are included into the plots.

Table 2
Affinity constants and protein binding of enantiomers of PRO and enantioselectivity to HSA.

Enantiomer	log K_1 [a]	PB [b]	ES [a]
E1	3.20 ± 0.16	48.7%	1.5 ± 0.3
E2	3.40 ± 0.14	60.1%	

[a] Robust estimates, median ± MADe (K_1 in M^{-1} units was calculated from Eq. (2)).
[b] PB = 100($D - d$)/D), where d is estimated from Eq. (3), for the range $D = 0.1$–1 μM and $P = 600$ μM.

ES). In fact, AFE1 and AFE2 follow a normal distribution according to the Shapiro–Wilk test ($P > 0.05$), but not *ES* ($P < 0.05$; although its standardised kurtosis value, −0.55, is within the range expected for data from a normal distribution). In view of the nature of data we decided to use robust statistics. Thus, the median and MADe (a robust estimation of the standard deviation [9]) were used as final estimates (see Table 2). Qualitatively, it becomes clear that E2 shows higher affinity to HSA than its antipode, so ES > 1 values are obtained (Fig. 4). However, since the differences are not too high, it is convenient to check this observation through hypothesis testing. Statistical comparison of enantiomers log K_1 paired data-vectors was performed. Both the parametric *t*-test for the means and the non-parametric ones for the medians (the sign test and the signed rank test) indicate significant differences ($P < 0.05$) between log K_{E1} and log K_{E2} data vectors (also between ES data vector and the unity), so significant enantioselectivity is demonstrated.

All the previous estimates come from a given model based on the $n_1 = 1$ assumption (1:1 stoichiometry for PRO enantiomers) and a main affinity site class in HSA for PRO; expected under *in vivo* conditions [6]. In order to confirm the stoichiometry assumption, the data were submitted to non-linear fitting based on Eq. (3), where SIMPLEX was used to fit the parameters n_1 and K_1. SIMPLEX searches were started with $n_1 = 1$, log $K_1 = 4$ for both enantiomers, and only integer n_1 values were allowed [6]. The log K_1 estimates found were: 3.23 and 3.41, for E1 and E2, respectively, which agree with values in Table 2. In addition, $n_1 = 1$ estimates were found for both enantiomers, verifying the previous assumption on stoichiometry. R^2 values from the SIMPLEX were 0.6 and 0.7, for E1 and E2 respectively. These values are relatively low, which reflects the data variability in relation to the intermediate precision conditions used (it should be noted that R^2 values increase notably, up to 0.9 if data from single session/fraction processed are used, but it is not the aim of the work). As an example of the model adequacy, Fig. 5 shows the results associated to E2 based on Eq. (3); the validation plot for d (Fig. 5a) and the d *vs.* D plot (Fig. 5b) superimposing on it the fitted model (the best SIMPLEX solution). This suggests that log K_1 estimates in Table 2 could be considered acceptable values, with the advantage over Eq. (3) estimates that uncertainty (here expressed

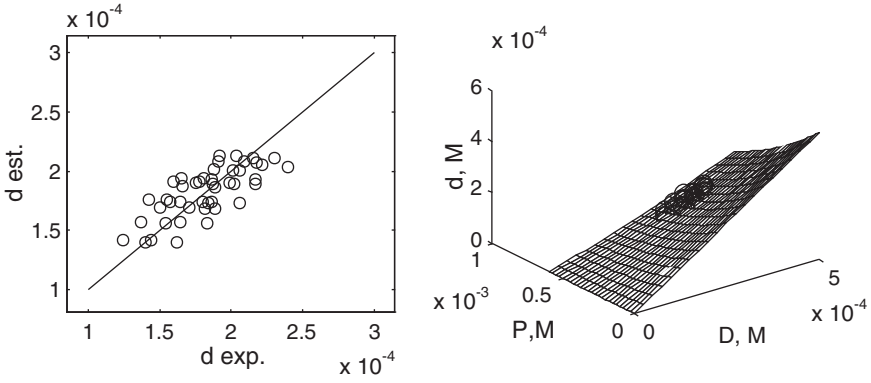

Fig. 5. Results from Eq. (3) (optimised by SIMPLEX) corresponding to E2. (a) Validation plot for d (estimated *vs.* experimental). (b) Response surface d *vs.* D and P; the experimental values are superimposed.

in terms of MADe, based on a minimum of 45 independent estimates from Eq. (2)) reflects the overall process variability under intermediate precision conditions including the day and processed fraction sources of variation. For the same reasons that $m = 1$ (and $n_1 = 1$) could be assumed, i.e. the high concentration of available binding sites at low D/P ratios [6], the probability of competitive binding between enantiomers should be low; therefore, no more complex and insecure [6] models seems to be necessary.

3.2.4. Protein binding estimation

As occurs with affinity or enantioselectivity information, previous *PB* data on PRO or its enantiomers has not been reported. Also, as far as we know, physiological concentration of PRO after its therapeutic use is not available in the literature. Normally, PB estimations corresponding to physiological conditions are preferred. They can be obtained from $PB = 100(D - d)/D$, where d can be estimated from Eq. (3), once n_1 and K_1 have been established, for available D and P *in vivo* data (e.g. $P = 600$ μM [6]). Due to the lack of *in vivo* D values, Table 2 includes estimations for D values in the 0.1–1 μM range. Lower PB estimates would be obtained for larger D values. These estimates are the first reported PB data for this pharmaceutical.

3.2.5. Final remarks

Unfortunately, the majority of the affinity and protein binding results obtained in the past come from a single approach, massive linear-regression plots derived from Eq. (1). These approaches are mathematically inconsistent [6] and require highly-accurate data to compensate for this drawback (in general incompatible with a process allowing enantioselective estimations from a racemate, such the one depicted here). Data comparison of results obtained from different approaches is at least recommendable, thus, the availability of experimental data in the publications (as in Table 1b) becomes essential to check alternative approaches such as Eq. (2), that allows all the potential of a univariate data treatment. Another aspect arises from experimental design, which in general does not take into account that high D/P ratios increase the risk of model assumptions invalidity (e.g. multiple binding, high-stoichiometry, competitive binding between enantiomers) or that intermediate precision conditions provide a more realistic uncertainty estimations concerning the published estimates. In view of the future work in this research area, this work is intended to influence the traditional schemes in order to improve the quality of the different processes involved in protein binding.

Most *in vitro* studies in the literature report affinity constants obtained using plain buffers (e.g. phosphate buffer during the incubation process, as here), without addition of salts (present in blood). However, the presence of salts in the buffer could displace the constants, so technically the calculated constants must be considered as conditional constants.

4. Conclusions

The *in vitro* incubation of racemic PRO–HSA mixtures at approximate physiological conditions, followed by ultrafiltration to separate/recover both the free and the bound fractions, and the separation/determination of enantiomers on both fractions by EKC has been applied to quantitatively evaluate, for the first time, the enantioselective binding of this pharmaceutical. Thus, mimicking *in vivo* experimental conditions, it is possible to access to information unapproachable by *in vivo* experiments (in this case, 1:1 stoichiometry, medium affinity constants, $\log K_{E1} = 3.20 \pm 0.16$ and $\log K_{E2} = 3.40 \pm 0.14$, and then protein binding percentage, $PB = 48.7$ and 60.1% for E1 and E2, respectively, and moderate but significant enantioselectivity, $ES = 1.5 \pm 0.3$, to the particular protein, HSA, the most relevant plasmatic protein in view of stereoselectivity).

From the results obtained, a simple direct equation can be recommended for individual estimates with further univariate statistics evaluation, facilitating the decision-making task and avoiding the traditional, insecure and less informative linear regression or non-linear fitting (the last suggested for verification purposes) approaches. In our opinion, the requisites of a high-affinity binding site ($m = 1$), 1:1 stoichiometry ($n_1 = 1$) and non-competitive model, compatible with this equation is consistent with the physiological levels of HSA and most drugs after normal dosage, but in practice implies that experiments must be performed keeping low D/P conditions. On the other hand, intermediate precision conditions (at least along two independent sessions) are preferable to guaranty reliable uncertainty estimations, consistent with the involved process complexity. In addition, data obtained from the free and bound fractions can be combined providing more reliable results and uncertainty.

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