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Short communication

Binding measurements of indolocarbazole derivatives to immobilised human serum albumin by high-performance liquid chromatography

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Abstract

The binding properties of six indolocarbazole derivatives have been measured using immobilised human serum albumin (HSA) in an HPLC column. The compounds showed very strong binding to HSA which necessitated the application of a 30 to 40% concentration of 2-propanol in the mobile phase. This represents a much higher concentration than is recommended by the column manufacturers. This HSA column had not changed its binding property when it was used again with 4% 2-propanol and 96% phosphate buffer. The binding parameters were estimated by extrapolation to 0% 2-propanol and were above 99% for each indolocarbazole derivative. The correlation analysis, including the calculated octanol/water partition coefficient ($\log P$), pK_a values as well as measured reversed-phase retention data of the compounds, revealed that the extremely strong binding can be explained by the hydrophobic and acidic properties of the compounds.

Keywords: Indolocarbazole derivatives

1. Introduction

The indolocarbazole derivatives represent a family of compounds with interesting antiviral properties. For the study of their in vivo and in vitro activity, binding to serum albumin is of potential importance. Many methods for the determination of drug protein-binding have been reported. Traditional methods involve equilibrium dialysis and ultrafiltration. These techniques are time consuming and the measurements are very difficult when the compounds show very strong binding to the serum albumin. Yoshida et al. [1] first suggested the application of HPLC for the determination of drug protein-binding using a physically coated ODS stationary phase. Good correlation of drug binding data has been reported by Lammers et al. [2], using a chemically bonded albumin stationary phase and equilibrium dialysis for 23 structurally heterogeneous groups of compounds. They found that the mobile phase with 3% 1-propanol eluted all of the compounds and did not influence the correlation to the binding data obtained by the equilibrium dialysis. Noctor et al. [3] reported excellent correlation between the drug binding per-

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centage (DB%) measured by ultrafiltration and the retention data ($k'/k'+1$) on HSA HPLC column for 19 benzodiazepines and 9 cumarins. Aubry and McGann [4] reviewed the application of HPLC using commercially available chemically bonded albumin stationary phases, and concluded that the application of less than 10% propanol did not seem to affect the specific binding. On the basis of quantitative structure–retention relationships on human serum albumin (HSA) Kaliszan et al. [5] characterised the binding site of the albumin with a hydrophobic pocket and a cationic region by which the more hydrophobic drugs with acidic functional groups can interact.

In our earlier study [6] on the acyclovir ester derivatives linear correlation was found between the drug binding data ($\log k'$) and the 2-propanol concentration in the mobile phase which ranged from 1 to 4%. In this study we report the need for the application of a much higher concentration of 2-propanol (30 to 40%) to elute the strongly bound indolocarbazole derivatives from this HSA column.

The relationship between the binding strength and calculated octanol/water partition coefficient ($\log P$) and pK_a values as well as with measured reversed-phase retention data is discussed.

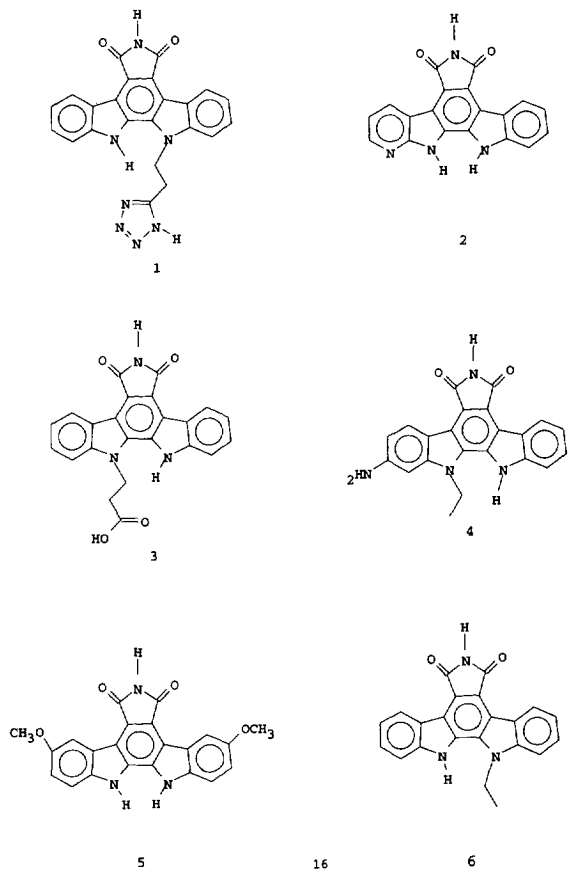

Fig. 1. Chemical structures of the investigated indolocarbazole derivatives.

2. Experimental

The chemical structures of the investigated indolocarbazole derivatives are presented in Fig. 1. The compounds were synthesised at the Wellcome Research Laboratories (Beckenham, UK) and were chromatographically pure. As the compounds have low solubility in water, saturated solutions were prepared in 2-propanol representing approximately 0.5 mg/ml concentration. These solutions were diluted with 20 m*M* phosphate buffer pH 7 in 1:3 proportion. An amount of 60 μl of the sample solution was injected onto the HPLC column.

The HPLC equipment consisted of two 510 Waters pumps with automated gradient controller, a Waters 712 WISP autosampler and Waters 490E programmable multiwavelength detector (Waters Division of Millipore, Milford, MA, USA).

An immobilised HSA column (Shandon, Runcorn, UK) 50×4.6 mm I.D. was used. The mobile phase was 20 m*M* phosphate buffer pH 7 and various concentrations of 2-propanol (30–40%). The flow-rate was 0.5 ml/min and the column was kept in a thermostatted oven at 25°C. Detection of the compounds was carried out at 235 and 340 nm UV with a sensitivity range of 0.2 absorbance unit full scale (AUFS). Quantitative evaluations of the chromatograms from the UV absorbance were made using a MULTICHROM data acquisition and analysis system (VG Data Systems, Altrincham, UK). The retention times (t_R) were the average of at least two measurements resulting from consecutive injections of the compounds onto the column. The dead time (t_0) value was measured by injecting a non-binding compound (acyclovir).

The drug binding percentage (DB%) was calculated from the retention time (t_R) values, through the retention factor (k') according to the equation below:

$$DB\% = \frac{(t_R - t_0)}{t_R} \cdot 100 = \frac{k'}{k'+1} \cdot 100$$

The extrapolation of the drug-binding parameter for the buffer (without 2-propanol) mobile phase was made by using the $\log k'$ values obtained at 30–35% 2-propanol concentration.

Reversed-phase retention data were measured on a Zorbax C_{18} Rx column (150×4.6 mm I.D.) (DuPont, Wilmington, DE, USA). The column was maintained at 30°C. The mobile phase flow-rate was 1.0 ml/min. The mobile phase was a mixture of 0.1 *M* ammonium acetate (pH 7.0) and methanol with the methanol concentration ranging from 70 to 90%. The HPLC-grade methanol and ammonium acetate were purchased from Rathburn (Loughborough, UK) and Fisons (Loughborough, UK), respectively. Water was obtained from a Milli-Q system (Millipore, CA, USA).

The octanol/water partition coefficient ($\log P$) and the pK_a values of the compounds were calculated by PALLAS for Windows program (CompuDrug Chemistry, Budapest, Hungary). The correlation analysis was carried out by Drugidea program (Budapest, Hungary).

3. Results and discussion

The compounds investigated could not be eluted in reasonable time from the HSA column by using less than 30% 2-propanol and buffer in the mobile phase.

Typical chromatograms are presented in Fig. 2 obtained by using 30% 2-propanol and 70% phosphate buffer (pH 7.0) as mobile phase. According to our earlier studies [6] the linear relationship between the $\log k'$ values and the 2-propanol concentrations can be supposed and the DB% parameters calculated from the extrapolated $\log k'_0$ values. The k' values, and the drug binding parameters (DB%) are shown in Table 1. It can be seen in Table 1 that all of the investigated compounds showed strong binding (above 99%) to HSA. The slope ($slope_{HSA}$) and the intercept ($\log k_{0,HSA}$) values of the straight lines obtained by plotting the $\log k$ values against the 2-propanol concentration are shown in Table 2.

The retention factors ($\log k$) measured by reversed-phase chromatography were plotted against the applied methanol concentration in the mobile phase. The slope ($slope_{RP}$) and the intercept ($\log k_{0,RP}$) values as well as the correlation coefficients are shown in Table 3. These values are generally accepted as the measure of hydrophobic character of the compounds [7]. Table 4 shows the calculated $\log P$ values and pK_a values for the compounds. Table 5 shows the correlation coefficients between the measured and calculated data of the six indolocarbazole derivatives. As opposed to our earlier studies on the acyclovir ester derivatives no significant correlation was found between the drug-binding data ($\log k_{0,HSA}$) and the $\log P$ values. The extremely strong binding cannot be explained by the $\log P$ values itself, as for example compounds from our earlier study [6] with $\log P$ values of 3 and 4.4 showed only 85.6% and 95.7% binding. Much higher correlation was found ($r=0.857$, see Table 5) between the binding data and the reversed-phase hydrophobicity parameter ($\log k_{0,RP}$). As the HSA binding sites contain a hydrophobic pocket and a cationic region [5], the pK_a values of the most acidic group were also taken into consideration as a second independent variable besides the hydrophobicity

Table 1
The measured drug binding data on HSA HPLC column (k' and DB%) for the indolocarbazole derivatives at various 2-propanol concentrations in the mobile phase

Compound	30% 2-Propanol		35% 2-Propanol		40% 2-Propanol		0% Extrapol.
	k'	DB (%)	k'	DB (%)	k'	DB (%)	DB (%)
1	7.08	87.6	2.43	70.8	1.40	58.3	99.98
2	4.77	82.7	1.99	66.7	1.33	57.2	99.89
3	6.65	87.1	2.43	70.8	1.38	58.0	99.96
4	4.06	80.2	2.11	67.9	1.23	55.3	99.55
5	4.88	82.9	2.14	68.1	1.33	57.1	99.86
6	6.03	85.8	2.36	70.2	1.33	57.2	99.94

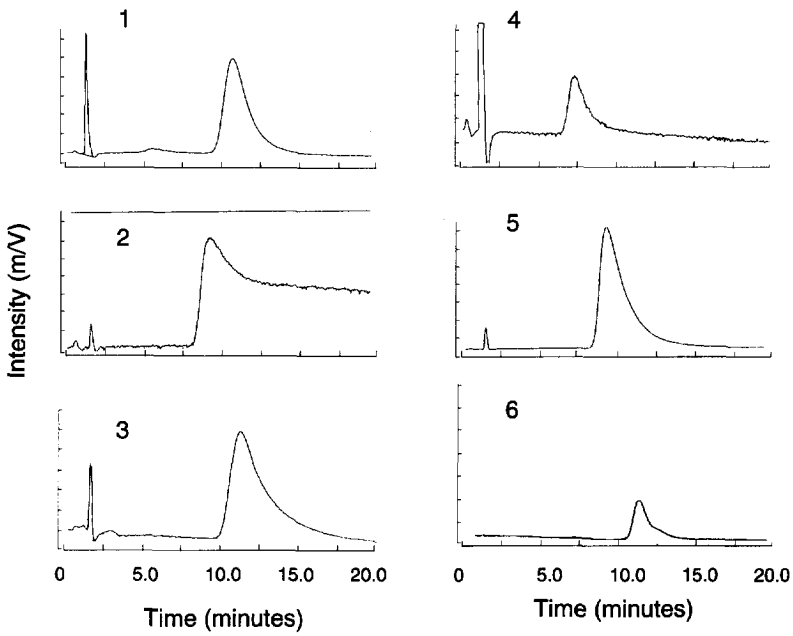

Fig. 2. Chromatograms of compounds 1 to 6 obtained on the immobilised HSA column (50×4.6 mm I.D.) with 30% (v/v) 2-propanol and 70% (v/v) phosphate buffer (pH 7). Flow-rate was 0.5 ml/min. Detection at 235 nm.

Table 2
Slope (slope$_{HSA}$) and intercept (log $k_{0,HSA}$) values of the straight lines obtained by plotting the measured log k values on HSA column against the 2-propanol concentration

Compound	Slope$_{HSA}$	log $k_{0,HSA}$
1	−0.0704	2.92
2	−0.0555	2.31
3	−0.0683	2.84
4	−0.0519	2.16
5	−0.0564	2.35
6	−0.0656	2.72

Table 3
Slope (slope$_{RP}$), intercept (log $k_{0,RP}$) and correlation coefficient (r) values of the straight lines obtained by plotting the reversed-phase log k values against the methanol concentration (n=5)

Compound	Slope$_{RP}$	log $k_{0,RP}$	r
1	−0.0533	4.05	0.999
2	−0.0401	3.31	0.999
3	−0.0493	4.28	0.999
4	−0.0443	3.32	0.999
5	−0.0477	3.90	0.999
6	−0.0486	4.24	0.998

Table 4
Calculated hydrophobicity (log P) and acidity (pK_a) values (1 for tetrazoyl, 2 for imide and 3 for carboxyl) for the indolocarbazole derivatives

Compound	log P	pK_a (1)	pK_a (2)	pK_a (3)
1	3.67	4.8	8.4	–
2	2.59	–	8.4	–
3	2.85	–	8.4	4.7
4	2.83	–	9.1	–
5	2.83	–	9.1	–
6	3.70	–	8.4	–

data. Significant relationships ($p<0.05$) were found between the binding data and both the hydrophobic parameters (log P and log $k_{0,RP}$) and the pK_a values of the most acidic group in the molecules as they are described in Eqs. (1) and (2).

$$\log k_{0,HSA} = 0.303(\pm 0.116)\log P - 0.106(\pm 0.027)pK_a + 2.407 \quad (1)$$

$$n = 6 \quad r = 0.958 \quad s = 0.118 \quad F = 16.5$$

Table 5
Correlation coefficients between the measured and calculated properties of the six indolocarbazole derivatives

	$Slope_{HSA}$	$\log k_{0,HSA}$	$Slope_{RP}$	$\log k_{0,RP}$	$\log P$	pK_a
$Slope_{HSA}$	1.000					
$\log k_{0,HSA}$	−0.999	1.000				
$Slope_{RP}$	0.815	−0.815	1.000			
$\log k_{0,RP}$	−0.856	0.857	−0.820	1.000		
$\log P$	−0.696	0.689	−0.742	0.620	1.000	
pK_a	0.850	−0.853	0.661	−0.857	0.850	1.000

$$\log k_{0,HSA} = 0.397(\pm 0.141) \log k_{0,RP} - 0.081(\pm 0.029)pK_a + 1.621 \quad (2)$$

$n = 6 \quad r = 0.962 \quad s = 0.111 \quad F = 18.5$

where n is the number of compounds, r is the correlation coefficient, s is the standard error of the estimate, F is the Fisher-test value.

It suggests that the strong binding of the investigated indolocarbazoles to HSA is specific and can be related to their hydrophobicity and/or acidic properties. Both equations suggest that by decreasing the hydrophobicity and/or the acidity of the compounds weaker binding to the HSA can be expected.

The column performance was checked after the measurements by using only 4% 2-propanol and injecting previously studied acyclovir esters. After two weeks of using the HSA column above 30% 2-propanol, no change in the binding properties could be observed, which suggests that the application of the high-organic solvent did not induce irreversible conformational change.

In conclusion, the investigated carbazole derivatives showed very strong binding to HSA. This unusual strong binding can be explained by the hydrophobic and acid-base character of compounds. The compounds could not be eluted from the HSA column by using less than 30% 2-propanol. The application of the unusual high percentage of organic solvent did not cause irreversible change in the binding properties of the immobilised HSA.

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