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Influence of Lipophilicity on the Protein Binding Affinity of Cephalosporins

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INTRODUCTION

The binding of antibiotics to plasma proteins, and particularly to albumin is a determinant factor in their pharmacokinetics, and hence in their therapeutic properties (1). The importance of this phenomenon has been shown especially in the case of cephalosporins (2,3). The cephalosporins, a chemically and pharmacologically homogeneous group (Table I), bind reversibly to human serum albumin (HSA) to different extents depending on changes in their side chain structure (4). Few experiments have been performed regarding the relationship between structure and binding affinity to HSA for cephem antibiotics in order to explain the nature of those interactions (4-8).

Some chromatographic methods, especially RP-HPLC, have been proposed to determine the lipophilicity of drugs (9). An interrelationship between the partition coefficient P and the chromatographic column capacity factor k′ in RP-HPLC has been established (10). We have already developed such a chromatographic approach to the hydrophobic properties of new diuretic compounds (11). In a recent review on hydrophobicity of β-lactam antibiobiotics, Petrauskas *et al.* (12) reported the difficulty in determining their lipophilicity by the classic shake flask method. Few studies have examined relationship between lipophilicity and binding affinity with HSA for cephalosporins (4,7). Tawara *et al.* (4) have evaluated the hydrophobic character by the retention time obtained with the same mobile phase for all tested cephem antibiotics.

The purpose of this paper is to study the lipophilicity of eleven cephalosporin derivatives. We measured the binding affinity of these compounds to HSA by equilibrium dialysis in order to establish a possible structure-binding relationship.

MATERIALS AND METHODS

Determination of Lipophilicity by RP-HPLC

Chemicals

Cephalothin and latamoxef (Lilly France SA, Saint-Cloud), cefonicid (SmithKline Beecham, Nanterre, France), cefoperazone (Pfizer Lab., Orsay, France), ceforanide (Bristol Lab., Paris-La Défense, France), cefotetan (Zeneca-Pharma, Cergy, France), cefotiam (Takeda Lab., Puteaux, France), cefoxitin (Merck Sharp & Dohme-Chibret, Paris, France), cefpiramide (Sanofi Winthrop, Gentilly, France), cephapirin (Bristol Lab., Paris La Défense, France), cefazolin (Allard Lab., Paris La Défense, France) were generously supplied from the cited companies and used without purification.

Apparatus and Chromatographic Conditions

Chromatography was performed with a Waters Assoc. apparatus equipped with a Model M 45 pump, a Lambda-Max Model ultraviolet detector operating at 270 nm and a Wisp Model automatic injector. The cephalosporins were chromatographied on a μBondapak C_{18} stainless-steel column (300mm × 3.9 mm, 10μm particle size) (Waters Assoc.). The mobile phase composition ranged from 20 to 80% (v/v) methanol with 0.06M phosphate buffer at pH 7.4. The flow rate was 1.5 ml/min. The detector output was recorded on a Data Jet integrator (Spectra Physics). All chemical and solvents were of analytical reagent or HPLC grade.

Determination of log k'_w Values

All drugs were of pharmaceutical purity. Stock solutions containing 1 mg/ml of the drug were prepared in methanol and subsequently diluted with water to the final injected concentrations (50 μg/ml). According to their chromatographic behaviour, the retention time (tr) of each cephalosporin was determined in triplicate at six different methanol-phosphate buffer mixtures. At each mobile phase composition, the measurement consisted in determining the capacity factor k′ calculated according to the formula k′ = (tr -to)/to, where tr and to were the retention times of the analyte and of the non-retained compound, respectively. The log k'_w values (logk′ at 100% aqueous mobile phase) were obtained from the y-intercept of plots logk′ *versus* percent of methanol in the mobile phase. The column dead-time of the system (to) was measured as the time from injection to the first distortion of the baseline after injection of each drug.

Protein Binding Studies

Chemicals and Solutions

The human albumin used was Fraction V essentially fatty acid-free from Sigma (St Louis, USA). All chemicals were of analytical reagent grade.

Equilibrium Dialysis: Experimental Conditions

All experiments were carried out at 37°C and all solutions were prepared with phosphate buffer (1/15 M, pH 7.4, with 50 mM NaCl).

Equilibrium dialysis was performed in a 2-ml macrocell using a rotative Dianorm apparatus (Braun Science Tec, Les Ulis, France). The two compartments were separated by a Spectra por 2 membrane (cutoff 12,000-14,000 D). In these

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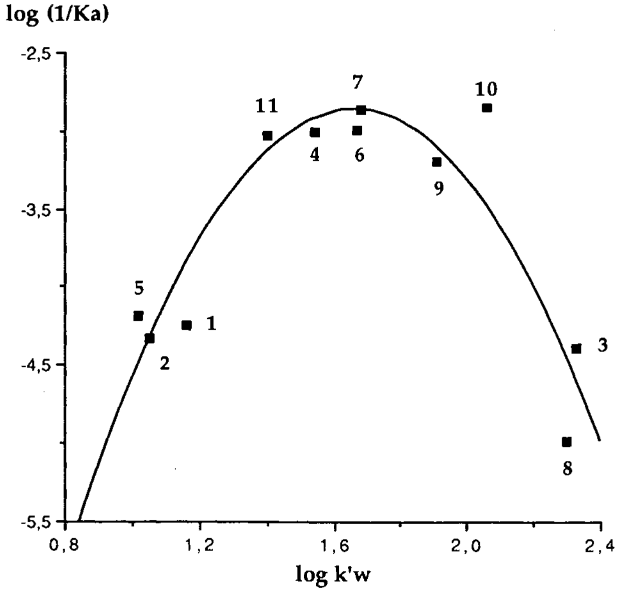


Fig. 1. Parabolic dependence of affinity constant Ka on lipophilicity log k'_w.

experimental conditions, 4 to 6h were required to ensure that equilibrium has been reached. The concentration of albumin was equal to 40g/l in each experiment, and the total antibiotic concentrations were between 20 and 600 μg/ml. The cephalosporins tested in this study were stable during the dialysis in both phosphate buffer and 4% HSA solution. Concentrations of total (T) and free drug (F) were determined by an HPLC assay as previously described (13). For each cephalosporin, an experiment was carried out without protein to verify the absence of adsorption on cell and membrane. Each percentage value of binding represents the average of three determinations.

Data Analysis

The binding of cephalosporins to albumin was analysed in terms of the classical model proposed by Scatchard.

The following equation was applied to determine the interaction parameters

$$r = nKaF/1 + KaF$$

where

n = number of binding sites;

Ka = association constant;

F = concentration of free drug;

r = number of moles of bound drug per mole of protein.

The existence of only one class of binding sites was considered. The plots were adjusted to a straight line by a least square regression analysis.

RESULTS

Lipophilicity of Cephalosporins: log k'_w Values

The lipophilicites were evaluated for these 11 cephalosporins by the chromatographic column capacity factor k' in a RP-HPLC system. For all compounds, linear relationships ($r > 0.98$) were proved to exist between the logk' values and the methanol percentages in the mobile phase, allowing the calculation of log k'_w. The log k'_w values obtained by regression analysis of logk'data and retained as the lipophilic indexes are listed in Table II. The log k'_w values ranged from 1.02 (cefotetan) to 2.33 (cefoperazone).

Binding of Cephalosporins to HSA: Determination of n and Ka

The Scatchard analysis was performed considering only one class of binding sites. Equilibrium constants (Ka) and number of binding sites (n) for eleven cephalosporins are shown in Table II. The values of Ka varied 140-fold among these studied cephem antibiotics. The number of binding sites per mole of albumin ranged from 0.67 (cefpiramide) to 2.71 (cefoxitin). Cefpiramide, a very highly bound cephalosporin (percentage of binding > 98%) exhibited the highest value of affinity constant ($Ka = 97286\ M^{-1}$) and the lowest value of number of binding site per mole of albumin ($n = 0.67$).

Relationship of Ka and k'_w for Cephalosporins

The relationship between lipophilicity (log k'_w) and the affinity constant (Ka) was assessed. As shown in Figure 1, a significant parabolic relationship ($r = 0.913$) was found between log k'_w (independent variable) and log (1/Ka) (dependent variable) values. The correlation which best fitted the experimental results corresponded to the following mathematical expression:

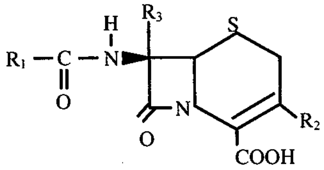
$$\begin{aligned} \log(1/Ka) &= -3.91\ (\pm 0.62)\ \log k'^{2}_{w} \\ &\quad + 13.01\ (+2.07)\ \log k'_w - 13.68 \\ n &= 11;\ r = 0.913; \\ \text{Fisher-test } F &= 20.06\ (p < 0.001) \end{aligned}$$

DISCUSSION

The extent of binding to HSA may be affected by the drug concentration, the protein concentration and the association constant (Ka). Since lipophilic forces are involved in protein binding, the hydrophobic character of a compound may also affect its binding affinity (14). To obtain further insight into the interrelationship between these two factors, we investigated the influence of lipophilicity on albumin binding of a series of cephalosporins.

The difficulty in determining the lipophilic parameter of β-lactam antibiotics has been emphasized in earlier reports (12). The generally accepted rules of hydrophobicity calculations suitable for others classes of compounds were thought to be inapplicable to cephalosporins. We have chosen to measure the lipophilicity by a previously described RP-HPLC method (11) and the hydrophobicity of 11 cephalosporins was estimated as log k'_w values. There are abundant reports in the literature exemplifiying excellent correlations between retention on RP-HPLC (log k'_w values) and measured or calculated octanol/water coefficients (logP) (9). Our binding parameters (n and Ka) are in good agreement with those previously published. For the measurement of these parameters, different methods are used: the centrifugal ultrafiltration method (7) or the equilibrium dialysis (5). In the experimental conditions of Briand (5) (HSA concentra-

Table I. Chemical Structure of Cephalosporins



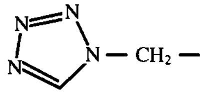
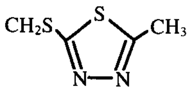
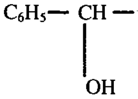
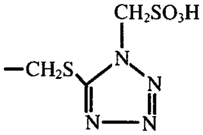
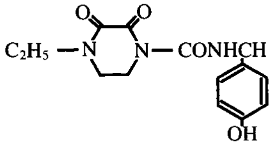
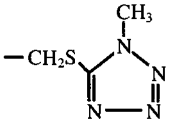
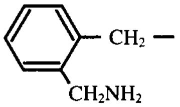
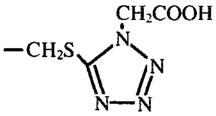
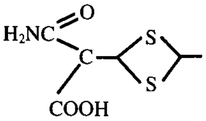
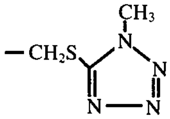
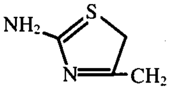
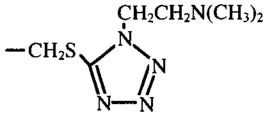
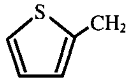
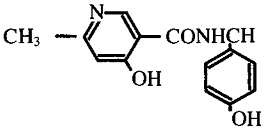
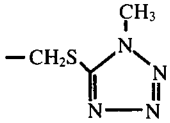
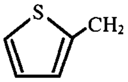
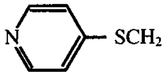
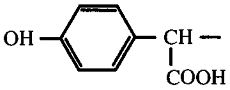
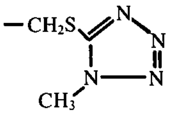
Compound	R1 (C7 side chain)	R2 (C3 side chain)	R3
Cefazolin			H
Cefonicid			H
Cefoperazone			H
Ceforanide			H
Cefotetan			OCH_3
Cefotiam			H
Cefoxitin		CH_2OCONH_2	OCH_3
Cefpiramide			H
Cephalotin		CH_2OCOCH_3	H
Cephapirin		CH_2OCOCH_3	H
Latamoxef			OCH_3

Table II. Log k'_w Values and Binding Parameters of Cephalosporins[a]

Compounds	log k'_w	n	Ka (M^{-1})
1-cefazolin	1.16	0.81	17469
2-cefonicid	1.05	1.13	21176
3-cefoperazone	2.33	0.61	24342
4-ceforanide	1.54	2.38	1002
5-cefotetan	1.02	0.93	15002
6-cefotiam	1.67	1.06	985
7-cefoxitin	1.68	2.71	729
8-cefpiramide	2.30	0.67	97286
9-cephalothin	1.91	2.62	1554
10-cephapirin	2.06	1.93	709
11-latamoxef	1.40	1.19	1079

[a] n: number of binding sites per mole of albumin. Ka: affinity constant.

tion: 34.5 g/l), cephapirin had only moderate affinity for HSA (Ka around 1000 M^{-1}) and cefazoline bound strongly (Ka value of 20000M^{-1}).

There are some reports on the structure-binding relationship of cephalosporins to HSA (4-7). Unfortunately, their results are contradictory. Tawara et al. (4) found a correlation between lipophilicity and the extent of HSA binding for cephalosporins with non-ionized C-3 side chain, and suggested that these compounds interact with albumin by ion binding or hydrophobic binding at site I (warfarin binding site). They explored the effect of C-7 side chain on the binding and concluded that this effect was less remarkable than that of C-3 side chain. Briand *et al.* (5) showed that binding forces involved in the interaction between cationic sites of HSA and some cephalosporins were principally electrostatic. Nouda et al. (6) and Terazaki et al. (7) reported that the correlation between lipophilicity (partition coefficient determined in isobutyl alcohol-pH 7.4 phosphate buffer system) and binding affinity of cephalosporins to HSA was very poor. Thus, other binding forces rather than the hydrophobic ones may play an important role for the HSA binding. They suggested that the absence of relationship between lipophilicity and HSA binding affinity would probably be ascribed to the presence of an additional binding force between the C-3 side chain of cephalosporins and an amino acid of HSA. Therefore, the characterization of binding sites and the nature of binding forces specific for cephem antibiotics to HSA were not completely elucidated.

Our results suggest that the binding affinity of cephalosporins to HSA depends on their lipophilic character. A significant parabolic relationship between the affinity constant log Ka and log k'_w was found. Compounds exhibiting either low or high lipophilicity (ie, log $k'_w < 1.2$ or > 2.2, respectively) present a strong affinity to albumin (Ka $>$ 15000M^{-1}). Six cephalosporins (ceforanide, cefotiam, cefoxitin, cephalothin, cephapirin and latamoxef) with log k'_w values between 1.2 and 2.2 have only a moderate affinity for HSA (Ka $<$ 1600 M^{-1}). This parabolic relationship exemplifies the complexity of the nature of binding forces to HSA. The hydrophobic interaction is a major driving force for protein binding of various drugs, increasing linear relationships between Ka and the selected lipophilic parameter are usually noted (15). The binding of these weak anionic compounds to the first class of sites are influenced by hydrophobic forces, but other forces may be involved.

Considering the other compounds (cefazolin, cefonicid, ceforanide, cefotetan and latamoxef), a decrease in binding affinity associated with an increase of lipophilicity was observed (Figure 1). This result suggests that other forces than lipophilic ones may take a prominent part in the binding phenomenon. Several studies have reported that probably cephalosporins interact with HSA by ion binding (4,7,8). The ionization behavior of these acidic compounds at physiological pH must be taken into account.

Further experiments are needed to characterize the respective contributions of these hydrophobic bonds and ionic forces in cephalosporin binding.

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