BIOLOGICALLY IMPORTANT PHYSICOCHEMICAL PROPERTIES OF SOME CEPHALOSPORINS

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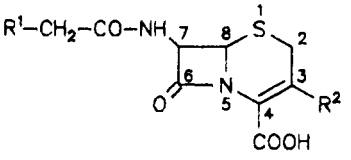
Received October 31, 1991
Accepted March 7, 1992

In the series of cephalosporin derivatives, consisting of eight 7-(R^1–CH_2–CO–NH)cephalosporanic acids and of seven analogical compounds with 3-acetoxymethyl replaced by 3-CH_3, physicochemical properties, which are expected to play a role in their antibacterial effects (the transport rate parameters and partition coefficients in the systems 1-octanol–water and 1-octanol–buffer, dissociation constants of the 4-carboxyl group, reactivity towards L-glutathione imitating the nucleophilic groups of the cell components and hydrolysis rate parameters), were determined. Linear dependences were observed between the partition coefficients and the π-constants of the varying substituents as well as between reactivity towards SH-groups of L-glutathione and OH-groups. The relationship between the transport rate parameters and partition coefficients, both measured in buffered as well as non-buffered system, was described by a common non-linear equation.

Cephalosporins like the penicillins are β-lactam antibiotics with a broad antibacterial spectrum, a low toxicity, and minimal undesirable secondary effects. Making use of the case of modification of the peripheral groups of the cephem nucleus chemists try to prepare new semisynthetic and synthetic cephalosporin derivatives with the aim of increasing antibacterial activity, broadening antibacterial spectrum and improving pharmacological properties. Till now several thousands of cephalosporin derivatives were synthesized but only about thirty of them are clinically used[1]. Taking into account the increasing number of resistant bacterial strains, the necessity of development of new derivatives is still actual.

The development of new bioactive derivatives can be rationalised using quantitative structure–activity relationships[2] (QSAR). In addition, these methods can provide a



deeper insight into the mechanism of action of the studied compounds. With the aim of obtaining suitable parameters for QSAR studies, physicochemical properties (acido-basicity, hydrophobicity, reactivity) of 15 cephalosporins (structure see above, the substituents R^1 and R^2 are given in Table I) were determined in this study.

EXPERIMENTAL

Chemicals

Cephalosporins *I*, *III* – *XV* (Table I) were synthetized[3,4] by one of the authors (M. V.) in the Drug Research Institute in Modra. Cephalosporin *II* is clinically used cephalotin (Biotika, Slovenská Lupča, Czechoslovakia). The infrared spectra were measured in KBr pellets. Stock solutions in dimethyl sulfoxide (DMSO) were used for all experiments. The final concetration of DMSO in the experiments never exceeded 1%. Other used chemicals were of analytical grade: reduced L-glutathione (Fluka, Buchs, Switzerland), 2,2'-dinitro-5,5'-dithiobenzoic acid (DTNB) (Serva, Heidelberg, Germany), 1-octanol, dimethyl sulfoxide, tris(hydroxymethyl)aminomethane (TRIS), acetonitrile, $(NH_4)_2SO_4$ (all Lachema, Brno, Czechoslovakia).

The Dissociation Constants

The values of dissociation constants were determined by the spectrophotometric titration at a constant wavelength[5]. A solution of the cephalosporin derivative in DMSO was diluted by distilled water to the final concentration $1 \cdot 10^{-4}$ mol dm^{-3}. At proper time intervals during the titration by 0.5M hydrochloric acid both the pH value of the mixture and its UV and VIS apsorption spectra were recorded. The dissociation constant was determined[5] from the pH-dependence of absorbance at the wavelenght of the absorption maximum.

Kinetics of the Reaction of Cephalosporins with L-Glutathione

Tripeptide L-glutathione (γ-L-glutamyl-L-cysteinyl-L-glycin) was used as the model nucleophile that imitates the nucleophilic groups of the cell components. The time course of the reaction in Tris-HCl buffer[6] (pH 8, with $1 \cdot 10^{-3}$ mol dm^{-3} EDTA to prevent L-glutathione from oxidation[7]) at 25 °C was monitored via determination of the free L-glutathione concentration using the Ellman method[7]. The starting concentration of L-glutathione was $3.6 \cdot 10^{-4}$ mol dm^{-3} and that of cephalosporins $1.4 \cdot 10^{-3}$ mol dm^{-3}. The cephalosporins were added to reaction mixture as a solution in DMSO. The final concentration of DMSO never exceeded 1 vol. %. The samples (0.5 ml) for determination of L-glutathione were withdrawn from the reaction mixture at proper time intervals and processed by addition of DTNB (0.5 ml, c = 4 mg ml^{-1}) and, after two minutes, of methanol (2 ml) for stopping the reaction. The concentration of the resulting 5-mercapto-2-nitrobenzoic acid was determined spectrophotometricaly at λ 412 nm (the absorption coefficient ε $1.36 \cdot 10^4$ mol^{-1} dm^3 cm^{-1}, ref.[7]). Under these conditions the reaction of cephalosporins with L-glutathione is of the second order and its time course can be described by ref.[8]

$$k_2 t = \ln \{c_C A / [c_G \varepsilon d (c_C - c_G) + A]\} / (c_G - c_C), \qquad (1)$$

where k_2 is rate constant of reaction of cephalosporins with L-glutathione, c_G and c_C are the initial concentrations of L-glutathione and cephalosporin, A is absorption at time t, ε the molar absorption coefficient, and d is the lenght of the light path. The rate constant k_2 was determined by linear regression analysis according to Eq. (*1*).

Kinetics of Alkaline Hydrolysis

Alkaline hydrolysis (1 mol dm^{-3} K_2HPO_4, pH 12, 25 °C) of cephalosporins ($1 . 10^{-4}$ mol dm^{-3}) was monitored by the changes in their UV and VIS absorption spectrum. The value of rate constant, k, was determined by linear regression analysis according to Eq. (*2*) which is valid for the first order reaction with absorbing substrate and product[8]

$$k\,t = \ln (A_0 - A_e) / (A - A_e), \qquad (2)$$

where A, A_0, and A_e represent the actual (at the time t), initial, and final absorbance at the wavelenght with maximal changes of spectra.

Interphase Distribution

The distribution behaviour of the cephalosporin derivates in the two-phase system 1-octanol–water (or 1-octanol–phosphate buffer according to Clark and Lubs[6], pH 7.4) were characterized by the transport rate parameters and by the partition coefficients. The measurements were made in a glass vessel[9] temperated to 25 °C. The stirring frequency (1.3 s^{-1}) was adjusted so as no concentration gradients could appear in either phase and the motion of the phase interface was minimal. The measuring vessel was filled with redistilled deionized 1-octanol satured with water (175 ml), temperated to 25 °C and surfaced with the solution of the respective compound in freshly distilled 1-octanol saturated with water or buffer (20 ml). Samples (2 ml) for spectrophotometric determination of the compound concentration were withdrawn from water phase with a syringe at proper time intervals. The UV spectra of the samples were recorded in the complete spectral range; this measurement also served for monitoring the stability of cephalosporins in the given medium. The samples were replaced to the vessel immediately after each measurement. Providing that at the beginning of partition the compound is present in 1-octanol only and its concentration is c_0, the time course of the concentration in aqueous phase (c_1) can be expressed by[9]

$$c_1 = c_0\, l_2\, V_2 \{1 - \exp [-S (l_1 / V_1 + l_2 / V_2)\, t]\} / (l_1 V_1 + l_2 V_2). \qquad (3)$$

Here l_1 and l_2 are the transport rate parameters in the direction from the aqueous phase to 1-octanol and backwards, respectively, V_1 and V_2 stand for the volumes of aqueous and 1-octanol phases, S for interface surface and t for time. The time course of the cephalosporin absorbance in the aqueous phase is expressed by Eq. (*3*) multiplied by εd (ε is the molar absorption coefficient, d is the length of the light path). Rearrangement of Eq. (*3*) as expressed by absorbance yields[9]:

$$A = \varepsilon\, d\, c_0\, l_2\, S\, t / V_1;\ t \to 0 \qquad (4)$$

$$\ln (A_e - A) = \text{const.} - S (l_1 / V_1 + l_2 / V_2)\, t, \qquad (5)$$

where A_e is the value for absorbance at the end of distribution. The values of transport rate parameters l_1 and l_2 were determined by nonlinear regression analysis[10] of the time course of cephalosporin absorbance in the aqueous phase according to Eq. (*3*) expressed through absorbance; Eqs (*4*) and (*5*) served for initial assesments of l_2 and l_1, respectively.

RESULTS AND DISCUSSION

Structure of the investigated compounds, the wavelenght of their absorption maximum, the molar absorption coefficients, the values of dissociation constants pK_a, the infrared

frequency ν(C=O), as well as the rate constants for the reaction with L-glutathione (k_{SH}) and for alkaline hydrolysis (k_{OH}) in aqueous solutions are summarized in Table I. The compounds can be subdivided into two groups according to the substituent in the position 3. The derivatives *I* – *VIII* (3-acetoxymethyl) have the pK_a values lower by 0.2 – 0.5 units than the compounds *IX* – *XV* (3-methyl) with the identical substituent in the position 7. All the investigated cephalosporins are totally dissociated under the physiological conditions.

As known, antibacterial effect of cephalosporins are elicited by their reactions with nucleophilic groups of the enzyme transpeptidase[11] which play a key role in the synthesis of cell walls. L-Glutathione was chosen as a reaction partner for the characterization of chemical reactivity, since it contains the free SH-group of cysteinyl, which can imitate nucleophilic groups of the enzyme. The rate constants of the alkaline hydrolysis were estimated in order to get insight into the stability of the tested compounds in the medium. The compounds containing the 3-acetoxymethyl substituent are more reactive than those with the 3-methyl substituent. The dependence of both acidobasicity and reactivity on structure of the studied compounds is more or less parallel, although the mutual correlations are not statistically significant (the correlation coefficients r in the two abovementioned groups are less than 0.8). The subdivision of the set into two groups reflects a larger inductive effect of the 3-methyl group in comparison with the 3-acetoxymethyl substituent. The substituents in the position 7 also influence the observed properties. The rate parameters for the reaction with L-glutathione (k_{SH}) and for alkaline hydrolysis (k_{OH}) are interdependent.

$$\log k_{SH} = (0.883 \pm 0.091) \log k_{OH} + (2.573 \pm 0.267)$$

$$n = 15,\ r = 0.950,\ s = 0.066,\ F = 106.4 \qquad (6)$$

In the whole manuscript n is the number of experimental points, s is the standard deviation and F is the value of the Fisher test. Thus, the reactivity towards the nucleophilic groups of the target enzyme and the stability in the medium, are mutually interrelated. This implies a non-linear dependence of biological activity on reactivity, because with growing reactivity a point could be reached, when the hydrolysis rate is high enough to diminish significantly the drug concentration before the receptor sites are modified. Investigation of distribution kinetics in a two-phase system is a suitable method for determination of the partition coefficient, especially in the case when the investigated compounds may undergo hydrolysis or other reactions[9]. This method provides, in addition to the check of stability of the compounds, an estimate of the rate of their distribution between nonpolar and aqueous phases, which can be of importance in connection with their passive transport in biological systems. The distribution properties of compound were characterized in two systems: 1-octanol–water and 1-octanol–buffer (pH 7.4). The results are presented in Table II. The partition coeffi-

TABLE I

Structures of cephalosporin derivatives *I* – *XV*, values of the wavelenght of absorption maximum λ (nm), the molar absorption coefficients ε (mol^{-1} dm^3 cm^{-1}) in distilled water, the dissociation constants pK_a, infrared frequency ν(C=O) (cm^{-1}) in the β-lactam nucleus, the rate constants towards L-glutathione k_{SH} (mol^{-1} dm^3 s^{-1}) and alkaline hydrolysis k_{OH} (s^{-1})

Compound	R^1	R^2	λ	ε . 10^{-4}	pK_a	ν	k_{SH}	$k_{OH} \cdot 10^4$
I	thienyl	CH_2OCOCH_3	263	0.921	2.30	1 775	0.716	7.413
II[a]	thienyl	CH_2OCOCH_3	263	0.831	2.35	1 753	0.845	7.943
III	furyl	CH_2OCOCH_3	263	0.922	2.20	1 775	0.695	10.00
IV	5-NO_2-furyl	CH_2OCOCH_3	278	0.914	2.50	1 785	0.716	8.337
V	5-C_6H_5-furyl	CH_2OCOCH_3	278	2.577	2.30	1 774	0.769	8.790
VI	4-Cl-C_6H_4-furyl	CH_2OCOCH_3	292	2.184	2.45	1 775	0.851	10.05
VII	2-Cl-C_6H_4-furyl	CH_2OCOCH_3	278	1.922	2.55	1 765	0.758	8.892
VIII	2,6-di-Cl-C_6H_3-furyl	CH_2OCOCH_3	263	1.416	2.65	1 785	1.000	11.22
IX	furyl	CH_3	263	0.753	2.80	1 765	0.343	4.932
X	5-NO_2-furyl	CH_3	278	1.768	2.65	1 785	0.332	3.083
XI	5-C_6H_5-furyl	CH_3	278	2.152	2.90	1 765	0.316	4.038
XII	4-Cl-C_6H_4-furyl	CH_3	292	1.998	3.00	1 765	0.394	3.715
XIII	2,6-di-Cl-C_6H_3-furyl	CH_3	263	1.022	3.10	1 775	0.345	3.115
XIV	thienyl	CH_3	263	1.104	2.85	1 760	0.315	4.036
XV[a]	thienyl	CH_3	263	0.843	2.85	1 760	0.309	3.083

[a] Sodium salt.

cients in the system with buffered aqueous phase encompass much broader range of the hydrophobicity scale than those measured in the 1-octanol–water system. The former quantities are in good correlation with the hydrophobicity of other compounds. This follows from Eq. (7), which represents the dependence of the experimental log P values of cephalosporins in the buffered system on the sum of empirically derived π-constants of varying substituents[12].

$$\log P = (0.965 \pm 0.124)\,\Sigma\pi - (1.487 \pm 0.162)$$

$$n = 15,\ r = 0.946,\ s = 0.415,\ F = 111.6 \qquad (7)$$

The hydrophobicities measured in 1-octanol–water system correlate neither with the π-constants nor with the log P values from the buffered system. Van de Waterbeemd and co-workers published a close relationship between the transport rate parameters and partition coefficients[13], which is valid not only for electroneutral molecules, but also for ionized species and ion pairs. As this finding is extremely important for an assessment of the drug distribution in biological systems, it was interesting to test whether our data follow the trends outlined in this study[13]. The dependence of the

TABLE II

The distribution properties of cephalosporins *I* – *XV* (structures in Table I) – the transport rate parameters in the direction water (buffer)–1-octanol (l_1) and backwards (l_2) in m s^{-1}, the partition coefficients $P = l_1 / l_2$, and the sum of π-constants of the varying substituents[12]

Compound	Redistilled water			Buffer (pH 7.4)			$\Sigma\pi$
	log P	$-\log l_1$	$-\log l_2$	log P	$-\log l_1$	$-\log l_2$	
I	0.199	3.199	4.193	0.563	3.840	4.431	1.78
II	0.173	3.989	4.200	0.554	3.847	4.440	1.78
III	–	–	–	0.091	4.101	4.199	1.31
IV	0.717	3.624	4.341	-0.185	4.301	4.115	1.03
V	-0.059	4.222	4.163	2.055	3.642	5.765	3.27
VI	0.722	3.761	4.483	2.760	3.590	6.462	3.98
VII	0.170	4.237	4.407	2.415	3.610	6.057	3.63
VIII	0.773	3.749	4.522	2.778	3.615	6.510	3.99
IX	-0.130	4.249	4.119	-0.295	4.457	4.101	1.70
X	0.748	3.992	4.740	-0.570	4.673	4.025	1.42
XI	0.290	4.041	4.331	1.661	3.616	5.331	3.66
XII	0.555	4.038	4.593	2.375	3.600	6.069	4.37
XIII	0.519	3.911	4.431	2.380	3.598	6.000	4.38
XIV	-0.104	4.181	4.076	0.179	4.059	4.215	2.17
XV	0.312	3.821	4.133	0.168	4.065	4.229	2.17

transport rate parameters l_1 and l_2 for the transfer of the solute from the aqueous phase to 1-octanol and backwards, respectively, upon the partition coefficient P can be expressed[13] as follows.

$$\log l_1 = \log P - \log [(0.350 \pm 0.121) P + 1] - (4.056 \pm 0.421)$$

$$n = 29, r = 0.967, s = 0.101, F = 51.4 \qquad (8)$$

$$\log l_2 = - \log [(0.500 \pm 0.198) P + 1] - (3.985 \pm 0.356)$$

$$n = 29, r = 0.967, s = 0.097, F = 73.4 \qquad (9)$$

A good agreement of the experimental data with Eqs (*8*) and (*9*) can be seen also in Fig. 1. Interestingly enough the data measured in both 1-octanol–water and 1-octanol–buffer systems can be combined and described by common Eqs (*8*) and (*9*). The data measured in the unbuffered system (empty points) show much narrower distribution along the log P scale as compared with those determined in the 1-octanol–buffer system (full points). Nevertheless, their adherence to the lines which represent Eqs (*8*) and (*9*) is quite satisfactory.

The set of physicochemical properties as given in Tables I and II can be used for derivation of model-based QSAR equations for description of structure non-specific processes (transport through the cell membranes, non-specific protein binding, spontaneous hydrolysis), which cephalosporins undergo in bacterial suspensions.

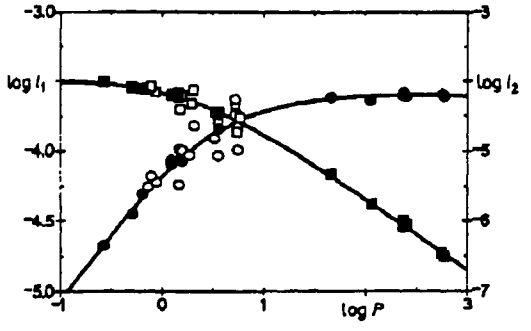


FIG. 1
The dependence of the transport rate constants in the direction from water (empty symbols) or buffer (full symbols) to 1-octanol (l_1 in m s^{-1}, circles) and backwards (l_2 in m s^{-1}, squares) on the corresponding 1-octanol–water or 1-octanol–buffer partition coefficient P of cephalosporins *I* – *XV*

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Translated by the author (Š. B.).