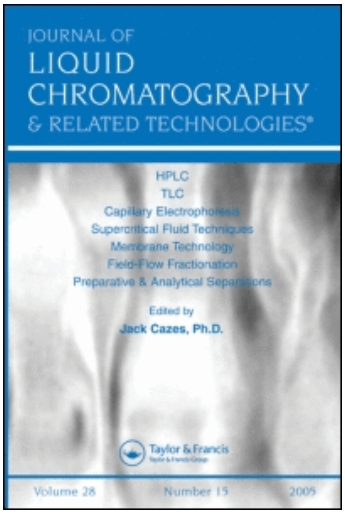

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CHARGE-TRANSFER CHROMATOGRAPHY USED TO STUDY THE INTERACTION OF CHLORHEXIDINE WITH PROTEINS AND AMINO ACIDS

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ABSTRACT

The interaction of the antibacterial agent chlorhexidine (1,1-hexamethylene-bis-/5-(4-chlorophenyl)-bisguanidine/) with human albumin and pepsin and with amino acids was studied with reversed-phase charge transfer chromatography in the presence of various monovalent cations. It was established that the mobility of chlorhexidine increases in the presence of ions in the lower concentration range (0.001 - 0.1 M) then increases at higher concentrations. The ion radii significantly influences the effect. Stepwise regression analysis proved that the chlorhexidine binds both to pepsin and human albumin, the binding is stronger with human albumin. The ions modify the character of chlorhexidine-human albumin binding indicating the hydrophilic character of the interaction. Except Asp and

Glu the other amino acids exhibited weak interactions with chlorhexidine, no significant difference was found between the interactive strength of L- and D-forms of Asp and Glu. Our data make probable that hydrophilic binding of chlorhexidine to the acidic side chains in proteins may be involved in its mode of action.

INTRODUCTION

Many studies have been devoted to the exploration of the effect of chlorhexidine (1,1-hexamethylene-bis-/5-(4-chlorophenyl)-bis-guanidine/) on the reduction of gingivitis (1-6) and plaque (7-12) formation. Chlorhexidine effectively reduces oral microflora (13-23) and the incidence of oral mucosal complications after bone marrow transplantation (24), inactivates human immunodeficiency virus (25), inhibits the incorporation in DNA of thymidine, decreases lactate dehydrogenase content in human buccal epithelial cells in culture (26) and desorbs adsorbed salivary constituents (27). However, chlorhexidine causes burns of the lips, mouth and tongue (28) and anaphylactic symptoms (29). Chlorhexidine interacts with surfactants (30), the addition of Triton-X-100 (31) or sodium lauryl sulphate (32) decreased its effect.

The mode of action of chlorhexidine has not been elucidated in detail. Chlorhexidine is thought to interact with acidic lipid components to cause changes in the membrane permeability (33), however, it changes only slightly the phase transition temperature of phosphatidylcholine and phosphatidylglycerol (34).

Charge-transfer reversed-phase thin-layer chromatography has been frequently used to study interactions between different organic molecules of low molecular

weight. The theory and practice of the determination of relative interactive strength by this method has also been described (35).

The objectives of our work were to study the interaction of chlorhexidine with some proteins and amino acids and to elucidate the effect of ions on the strength of interaction.

MATERIALS AND METHODS

Chlorhexidine diacetate (Serva Chemical Co.. USA) was of analytical purity. Human albumin and pepsin were the products of Human Vaccine Works (Budapest, Hungary) and Chemical Works of Gedeon Richter Ltd. (Budapest, Hungary), respectively. Amino acids were of analytical purity and of L-conformation, the D-forms were included in the experiments only in the case of Asp and Glu. To study the interaction of chlorhexidine with the proteins cellulose powder for TLC (Merck, Darmstadt, Germany) was mixed with the proteins in 9:1, 19:1 and 99:1 w/w ratios. Layers of 20 x 20 cm (0.25 mm thickness) were prepared from the mixed slurries and after drying their UV spectra was determined with a Model CS-930 Dual Wavelength TLC Scanner (Shimadzu, Kyoto, Japan). The UV spectra of chlorhexidine adsorbed on cellulose layer was determined in separate experiment. As the chlorhexidine was easily detectable even at the highest protein:cellulose ratio (1:9 w/w), this mixed sorbent was used in the experiments to study the interaction between chlorhexidine and the proteins. The use of unimpregnated cellulose layer as reversed-phase sorbent was motivated by the theoretical considerations stating that any layer may

behave as a reversed-phase one when the stationary phase is less polar than the mobile phase (36). The validity of the hypothesis outlined above was proved to be true for unimpregnated cellulose (37). Chlorhexidine diacetate was dissolved in distilled water at the concentration of 2 mg/ml, 5 μl of this solution was spotted onto the plates. Distilled water was used as eluent containing LiCl, NaCl and KCl in the concentration range of 0.001 - 1 mM. At each salt concentration unmixed cellulose layers served as control. After development the plates were dried at 105°C and the maximum of the chlorhexidine spot was determined with the same TLC scanner at 270 nm. The R_M values characterizing the lipophilicity were calculated in each case.

As it was assumed that the protein and salt concentration and the interaction between proteins and salts may influence simultaneously the R_M value of chlorhexidine we used stepwise resression analysis to elucidate this problem (38). As the exact type of correlation (linear, quadratic or logarithmic) between the independent and dependent variables was not previously established we used the following independent variables: x_1 = human albumin content of the sorbent (%); x_2 = pepsin content of the sorbent (%); x_3 = cation radii; $x_4 = (\log x_6)^2$; $x_5 = (x_1.x_7)^2$; $x_6 = (x_7)^2$; x_7 = salt concentration in the eluent (M); $x_8 = x_1.x_7$; $x_9 = x_2.x_7$; $x_{10} = (x_2.x_7)^2$; $x_{11} = \log x_7$. The number of accepted variables was not limited, the significance level for each independent variables was set to 95%.

To study the interaction of chlorhexidine with amino acids DC-Alufolien cellulose plates (Merck, Darmstadt, Germany) were used without any pretreatment.

Chlorhexidine was spotted onto the plates as described above. For the screening test distilled water was used as the eluent containing the amino acids at 10 mM concentration. The detection of the spot maximum was carried out by the TLC scanner as described above. As Asp and Glu showed the highest effect their interaction with chlorhexidine was studied more in detail, the concentrations of Asp and Glu varying between 0 - 10 and 0 - 100 mM depending on their solubilitv in water. To elucidate the role of stereospecificity the experiments with Asp and Glu were carried out with the L- and D-forms too. As the correlation between the Asp and Glu concentration and the R_M value of chlorhexidine seemed to be markedly nonlinear, logarithmic correlations were calculated between the R_M value and amino acid concentration separately for the L- and D-forms of Asp and Glu.

RESULTS AND DISCUSSION

The UV spectra of chlorhexidine and human albumin adsorbed on cellulose are shown in Fig.1. The chlorhexidine has an UV maximum at 270 nm. The human albumin (and also pepsin) has a relatively low absorbance at this wavelength, therefore the adsorbed proteins interfere to a negligible extent with the UV detection of chlorhexidine.

The lipophilicity of chlorhexidine decreasad rapidly with the growing concentration of salt in the eluent (Fig.2) then - having a minimum - increased again at higher salt concentrations. However, this increasing phase was slower than the decreasing phase. The effect was similar for each salt but depended also

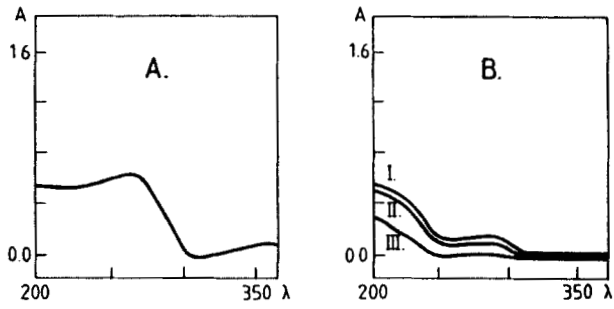

FIGURE 1. UV spectra of chlorhexidine (A) and human albumin (B) adsorbed on cellulose. I. human albumin: cellulose 1.9 w/w; II. human albumin:cellulose 1:19 w/w; III. human albumin:cellulose 1:99 w/w

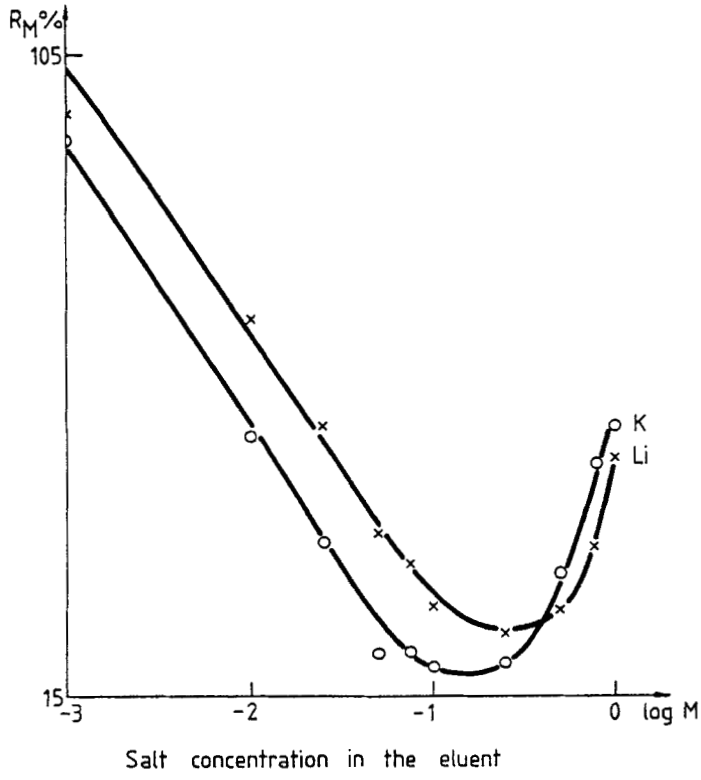

FIGURE 2. Effect of various salts on the R_M value of chlorhexidine.

on the type of salt. Our data make obvious that the salt in the eluent influences the R_M value of chlorhexidine in minimally two different ways. We assume that the polar groups of chlorhexidine interact with the polar adsorptive site of cellulose. The ions present in the eluent also bind to the polar adsorption centers of cellulose. This coadsorption is of competitive character, the higher the ion strength the higher is the ratio of adsorptive site occupied by the ions in the eluent. The lessening number of binding sites results in continuous decrease of chlorhexidine retention. Similar masking effects of eluent additives was also observed in the reversed-phase chromatography of peptides (39). After the probable saturation of free adsorptive sites of cellulose by the ions in the eluent the situation changes markedly. The fact that higher salt concentrations increase again the R_M values of chlorhexidine may be due to the dissociation suppressing effect of salts. The undissociated chlorhexidine molecule shows a higher reversed-phase retention than the dissociated one. Similar anomalous effect of salts on the reversed-phase retention behavior of heterocyclic quaternary ammonium salts has been previously reported (40).

The proteins markedly decreased the retention of chlorhexidine at higher salt concentration (Fig.3) indicating that the proteins really interact with chlorhexidine.

The results of stepwise regression analysis are compiled in Table 1.

The equation fits well to the experimental data the significance level being over 99.9% (compare calculated and tabulated F values). Six independent variables

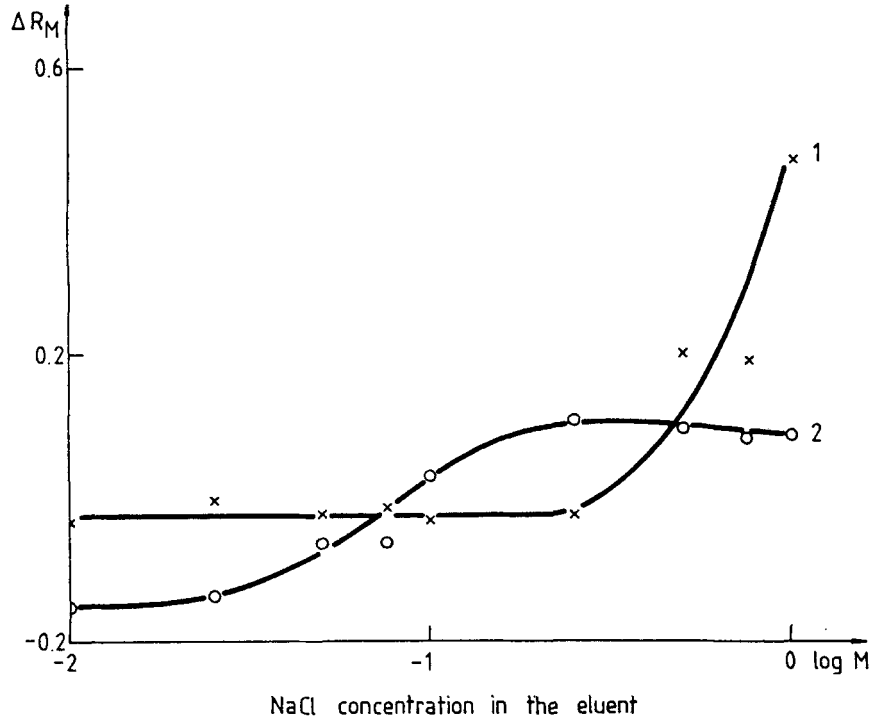

FIGURE 3. Effect of human albumin (1) and pepsin (2) on the lipophilicity of chlorhexidine.

of the eleven included ones account for about 74% of the total variance (see r^2 value). It can be concluded from the slope (b) values that both human albumin and pepsin significantly increase the retention of chlorhexidine that proves the binding of chlorhexidine to these proteins. The ions with greater ion radii decrease more strongly the retention of chlorhexidine that is not only the ion concentration but also the character of the cation influences the lipophilicity

TABLE 1.
Effect of Salts and Proteins on the Lipophilicity (R_M) of Chlorhexidine. Results of Stepwise Regression Analysis.

$$R_M = a + b_1.x_1 + b_2.x_2 + b_3.x_3 + b_4.(\log x_7)^2 + b_5.x_1.x_7 + b_6.(x_7)^2$$

$n = 74$ $a = 0.52$ $F_{calc.} = 32.59$ $F_{99.9\%} = 4.37$ $r^2 = 0.7420$

Number of independent variable	$b.10^2$	$s_b.10^3$	Path coefficient %
1	2.10	5.16	12.4
2	1.27	5.26	6.0
3	-21.97	10.73	5.0
4	10.55	8.50	33.5
5	-0.46	1.28	11.9
6	74.72	75.07	30.9

x_1 = human serum albumin content of the sorbent (%)
x_2 = pepsin content of the sorbent (%)
x_3 = cation radii
x_7 = salt concentration in the eluent (M)

of chlorhexidine. The exact type of correlation between the salt concentration in the eluent and the R_M value of chlorhexidine is not clear. Our data suggest that it can be quadratic or log quadratic. This finding supports our previous assumption that the influence of salts is composed of more than one physicochemical processes which are yet not clearly understood. The interaction between human albumin and salt concentration decreases the R_M value of chlorhexidine proving again the hydrophilic character of protein-chlorhexidine interaction. The path coefficients show the relative impact of the independent variables on the R_M value. The salt concentration exerts the highest effect on the retention followed by the pro-

TABLE 2.
Effect of Amino Acids on the Lipophilicity of Chlorhexidine.

Amino acid	Lipophilicity (R_M value) Mean	Standard deviation	Coefficient of variation%
Control	1.35	0.03	2.57
Ala	1.29	0.03	2.41
Arg	1.03	0.04	3.88
Asn	1.14	0.02	1.91
Asp	0.28	0.03	11.81
Cys	1.22	0.02	1.41
Glu	0.33	0.03	5.47
Gly	1.25	0.02	1.64
Ile	1.15	0.08	7.35
Leu	1.12	0.01	0.52
nor-Leu	1.09	0.10	9.30
Lys	1.09	0.05	4.18
Orn	1.04	0.02	2.24
Pro	1.36	0.02	1.54
Ser	1.34	0.03	2.57
Trp	1.40	0.05	3.57
Tyr	1.30	0.02	1.21
Val	1.29	0.04	3.10

tein concentrations. The impact of the ion radii is of secondary importance.

The results of the screening test for amino acid-chlorhexidine interaction are compiled in Table 2. Many amino acids decrease the lipophilicity of chlorhexidine. This phenomena is probably due to the fact that the more hydrophilic amino acids interact with the chlorhexidine reducing its lipophilicity. The highest effect was observed with the dicarboxylic amino acids Asp and Glu indicating that the interaction is of hydrophilic character.

We assume that the quaternary amino groups of chlorhexidine form hydrogen bonds with the carboxyl

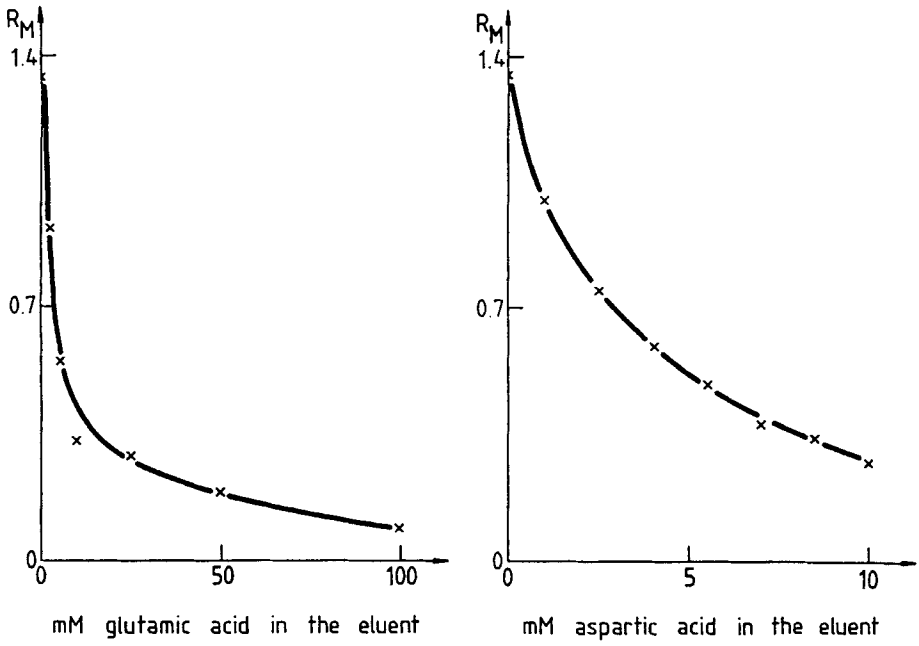

FIGURE 4. Effect of dicarboxylic amino acids on the R_M value of chlorhexidine.

groups of the amino acids resulting in charge transfer complexes of unknown stoichiometry. Unfortunately, charge transfer chromatography does not give information about the composition of the complexes. The correlation between the concentration of dibasic amino acids in the eluent and the R_M value of chlorhexidine is markedly nonlinear (Fig.4). As in the majority of cases the correlation between the two complexing molecules is significantly linear (41,42), this finding can be tentatively explained by the assumption that the complexes may have minimally two stoichiometries

TABLE 3.
Effect of Dicarboxylic Amino Acids on the Lipophilicity (R_M value) of Chlorhexidine (C = Concentration of Amino Acid mM).

$$R_M = a + b.\log C$$

Amino acid	n	a	b	s_b	$r_{calc.}$	$r_{99\%}$
L-Glu	5	0.91	-0.52	0.05	0.9795	0.9587
D-Glu	5	0.99	-0.57	0.03	0.9812	0.9587
L-AsP	7	1.02	-0.73	0.02	0.9979	0.9507
D-Asp	7	1.10	-0.70	0.04	0.9813	0.9507

depending on the amino acid - chlorhexidine ratio. At low amino acid concentrations the amino acid - chlorhexidine ratio is lower in the complex than at higher concentrations resulting in the deviation from the linearity.

Calculations show that the logarithmic correlation fits well to the experimental data the significance level being in each case over 99% (Table 3).

The change of amino acid concentration accounts for about 95 - 99% of the lipophilicity change of chlorhexidine (see r values). No significant difference was found between the slope (b) values of L- and D-forms that is the interaction of chlorhexidine with Asp and Glu not stereospecific or the stereospecificity is such low that it is under the detection limit of our method. The L- and D-forms of Asp had a significantly higher effect on the R_M value of chlorhexidine than the corresponding forms of Glu (the calculated t values were 3.32 and 2.60 for L- and D-forms, respectively, the tabulated t value for 95% significance level is 2.23). It means that chlorhexidine preferably

interacts with the more acidic (43) and less lipophilic (44) Asp proving again the hydrophilic character of the interaction.

Our results prove that chlorhexidine can bind to various proteins, the binding sites are probably the acidic amino acid side chains in the proteins. The interaction is of hydrophilic character and its strength depends on the type and concentration of the ions in the environment. We assume that these protein - chlorhexidine interactions may have a role in the biological activity of chlorhexidine.

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