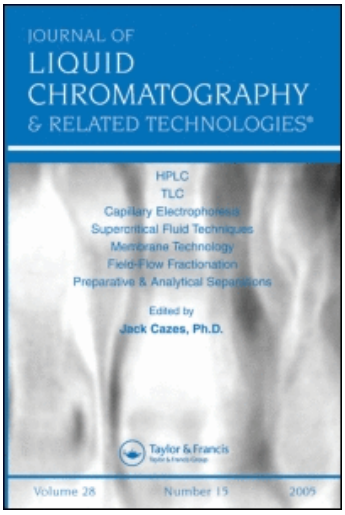

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HPLC Determination of pKa of Parabens and Investigation on their Lipophilicity Parameters

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Abstract: An HPLC method is applied for determination of the pKa values and lipophilicity parameter of the most useful preservatives in drugs oral solution formulations at different pH ranges. The same method is used for investigation on the lipophilicity of these compounds. The pKa and lipophilicity parameter values are the most important parameters characterizing the processes of penetration of the preservatives through the cellular membrane at different conditions. The higher lipophilicity contributes to better interaction of the preservatives with the membrane of undesirable microorganisms and assures their biological action.

Keywords: Preservatives, pKa value, Lipophilicity, Methylparaben, Propylparben, Parabens

INTRODUCTION

Preservatives are used in a wide range of applications maintaining overall product quality. They can be found in foods, beverages, pharmaceuticals, and personal care products. Three primary classes of preservatives are known, antimicrobials, antioxidants, and chelating agents.[1] Some of them are naturally occurring compounds.[2] Antimicrobials are added to pharmaceutical products to prolong shelf life and maintain sterility. Some act on yeasts, molds, and bacteria, while others specifically target certain classes

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of microbes. In the current work, some of the most common antimicrobial agents such as parabens (methylparaben, ethylparaben, propylparaben and butylparaben), which are being used in drugs and cosmetics because of their broad antimicrobial spectrum with good stability and non-volatility, will be investigated Figure 1.

Parabens may be considered as the most suitable preservatives for submicron emulsions, bearing in mind their visual characteristics, oily droplet sizes and pH value. Parabens are preservatives with the best physico-chemical compatibility with submicron emulsion within a broad range of pH: 5.0–8.2.[3]

The ability of a chemical to act as a preservative depends very much on the environment, therefore, factors such as type of the product, water content, pH, and storage conditions need to be considered when selecting preservatives.[4] Usually, better antimicrobial activity can be achieved using a combination of agents. Very often, a mixture of methyl paraben and propyl paraben is added to aqueous formulations because of their claimed synergistic effect.[3,5] These substances can have multiple biological effects, but it is generally considered that their inhibitory effects on membrane transport and mitochondrial function processes are key for their actions.[6] The lipophilicity of the compounds defined as its relative tendency to be readily soluble in most non-polar solvents but only partially soluble in water, plays an important role from a biological and environmental point of view. This is why determining the interaction of a compound with a lipophilic membrane is very important. As an evaluation of this interaction, the lipophilicity parameter of the substances investigated is considered.

Improved understanding of the relationship between structure and antimicrobial activity allowed development of new methods for their investigation.

Lipophilicity is usually expressed by the partition logarithm coefficient log *P* derived from studies of a compound distribution between water and an immiscible non-polar compound using the shake flask technique. Based on an extensive work of Hansch's group, it is now generally accepted that log *P* values obtained from an *n*-octanol-water partition system are particularly suitable for characterizing the interactions between chemical substances and the biological system.[7,8] This method was chosen after considering such properties as density, viscosity, dielectric constant, and mutual

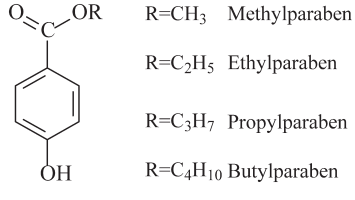

Figure 1. Structural formulas.

solubility with water.[9] In particular, the water saturated octanolic phase (which exhibits an octanol/water molar ratio close to 4/1) is highly saturated, each molecule of water being hydrogen bonded to four molecules of octanol.[9] *N*-octanol is, however, an isotropic liquid and this is in contrast to the strong anisotropic nature of a typical biomembrane for which *n*-octanol is used as a model. The major components of most membranes are phospholipids and cholesterol molecules forming a bilayer in which proteins and other lipids are incorporated.[7] Three structural different regions constitute the permeation barrier for bioactive solutes. The outer region is highly polar, composed from charged phospholipids heads groups. The middle region is medium polar and highly inflexible, composed from water a organic phase interface consisting tightly packet cholesterol rings, glycerol backbones, and the first few methylene units of the hydrocarbon chains of the phospholipids. The most inner region is extremely non-polar, flexible, and loosely packed, composed from the tail groups of the hydrocarbon chains. Thus, the membrane will not behave as a bulk liquid.

There are a number of similarities between the mobile phase–stationary phase interface in the RP-HPLC and the membrane–water interface. The chemically bonded phase does not behave as a liquid, but resembles much more the ordered array of the membrane hydrocarbon chains. The residual silanol groups, some of them being charged at neutral pH, and the adsorbed layer of hydrogen bonding organic modifier and coextracted water molecules may be expected to figure the polar, outer membrane regions. Finally, both systems are apparently in a dynamic state where true equilibrium is seldom achieved.[7]

The molecules of preservatives would move between "aqueous" and a variety of different "more or less organic" phases, going through the wall membrane section, then the endoplasmic reticulum, and finally (in many but not all cases) the membrane structure of a particular organelle. Knowing that, the lipophilicity parameter expresses a moving of a derivative form of preservatives molecules from one phase to another.[10] That is a demonstration of the dynamic nature of the processes through cellular membrane.

The similarities between lipophilicity parameter log P and the logarithm of the retention factor k' in HPLC and their approximation (as mentioned above), are the reason for using the method of RP-HPLC for determination of log P. In the literature, correlation between lipophilicity parameter P and the retention factor k'[8,11] is described. The retention factor was calculated from the peaks of the analyzed and non-retained compounds according to the following equation:

$$k' = \frac{n_s}{n_m} = \frac{(t_R - t_0)}{t_0} \tag{1}$$

where k' is the capacity factor, n_s and n_m denotes the numbers of moles of analyte in the stationary (s) and in mobile (m) phase, t_R is the retention time

of the compound investigated, and t_0 is the retention time of the unretained compound.[9]

Several studies have been reported on the variation of k' values of samples in isocratic reversed phase liquid chromatography, as a function of mobile phase consumption.[12,13] For mobile phases consisting of water or buffer and organic solvent, most often acetonitrile or methanol, it is usually observed for a given system, that sample k' values are related to the volume fraction of the organic solvent φ in the mobile phase.[7,9,14]

$$\log k' = \log k_w - S\varphi \tag{2}$$

where k_w refers to the isocratic k' value for pure water as mobile phase, and is usually extrapolated value, S is related to the solvent strength of pure organic modifier as mobile phase and is specific to this solvent on the stationary phase, φ is the volume fraction of the organic solvent in the mobile phase.[14]

In reversed phase high performance liquid chromatography (RP-HPLC) the chromatographic retention is governed by hydrophobic forces, and therefore various RP-HPLC retention data have been suggested for calculating the log P value of compounds.[15] There are three main approaches. The first is the use of RP-HPLC log k' values obtained on a given column with a given mobile phase consumption. The second approach is to use log k' values extrapolated to 0% organic modifier concentration (log k_w). The log k_w values can be directly obtained only for a relatively small number of compounds, and therefore, sometimes, a predicted value should be used. The third approach suggests a backwards extrapolation method for the log k' values, referring to an optimum organic phase concentration by which the 1-octanol-water partition system can be best modeled.[15] In this study, the first approach was chosen due to the following reasons.

The variety of interpretations of the relationship between log k' and ϕ values (linear or quadratic) could be avoided.[15]

The very good to excellent correlation between log P and log k' for the compounds investigated (R^2 was 0.9981 Figure 4) is evidence that the 1-octanol-water partition system is a very good model. It is suggested, that there is not an essential difference to whether we use the first (of the three mentioned above) approach instead of the second, or the third one.

The intensive use of HPLC for determination of lipophilicity is due to the fact that the technique presented, when using appropriate stationary and mobile phase, may produce retention data with accuracy better than the previously used (log P values obtained from n-octanol-water partition system).

The retention factor k' depends on the retention time t_R, related very often to the pH at which the chromatographic investigation is being performed. It is, therefore, important to define the pH range at which the retention factor has the value suitable for a certain preservative applications.

Using a C_{18} RP column and mixture of organic solvent and buffers with different pH values as mobile phase, the pH dependency of the capacity factors of four preservatives was investigated.

The method above is suitable for the determination of the lipophilicity parameter and pKa value of a preservative, in cases where the dependency of t_R to pH of the compounds investigated, is susceptible enough for the RP-HPLC investigation. As the t_R of parabens strongly depends on pH, especially at higher pH ranges (over 7.5), the retention factor k' and their distribution coefficient also depend on pH, and this dependency can be fixed for the conditions of the chromatographic investigation performed here.

EXPERIMENTAL

Reagents

Acetonitrile gradient grade, LiChrosolv-Merck, *n*-octanol, potassium phosphate, phosphoric acid, methylparaben, ethylparaben, propylparaben, and butylparaben are purchased from Merck and used as received.

Buffer Preparation

0.02 M potassium phosphate was adjusted with phosphoric acid.

Chromatographic Conditions

HPLC Integrated System Shimadzu LC2010 A equipped with chromatographic software Class VP 6.0 was used. RP column C_{18} (10 μm, 250 mm × 4.6 i.d), mobile phase: phosphate buffer (0.02 M potassium phosphate), acetonitrile 65/35 (v/v), flow rate 1.5 mL min^{-1}, injection volume 10 μL, $\lambda = 254$ nm. The different pH ranges of the mobile phase (2.0–9.45) were obtained by adjustment with phosphoric acid. As a non-retained compound, the peak of methanol was used. Spectrophotometer SF-46 (of USSR origin).

RESULTS

As a first step of the antimicrobial activity of the preservatives their penetration through a cellular membrane was considered. That is the reason why a determination of their lipophilicity parameter was carried out. Preservatives could have different penetration through a cellular membrane according to their own pKa values and pH of the environment. The distribution coefficient, which describes pH dependency of partitioning of the compounds

investigated between membrane and environment is strongly related to the pKa values and the environmental pH. Different values at different pH ranges could be obtained because of possible full or partial deprotonation or protonation of the suitable functional groups in the molecule structure of the preservatives. For compounds with only one ionizable group, the plot "distribution coefficient/pH" is usually a sigmoid curve.[9] The inflection point of this curve is very close to the pKa value of the compound investigated.[9] The plots of correlations between log k' and the different pH values for the parabens (compounds with only one ionizable group) are very similar to the plot "distribution coefficient/pH". Some of the best fits for the correlation obtained are equations of Pearson and Asymmetric Sigmoid. We choose one of the private cases of the equation of Asymmetric Sigmoid, which describes, in a very good way, the correlation found and is one of the simplest opportunities (Figure 2).

The coefficients of the following equation expressed the dependency between log k' of parabens and pH, and are presented in Table 1.

$$y = C + \frac{A}{[1 + \exp((x - D)^{*}K)]^{100}} \qquad D \neq 0 \tag{3}$$

Calculating the second derivative of the equation above for the curves presented on the graphs, we determined the inflection point, which corresponds to pKa value of the compounds investigated.

The data obtained for the pKa value are compared in Table 2 with the literature pKa values[16] and with the data calculated with well known

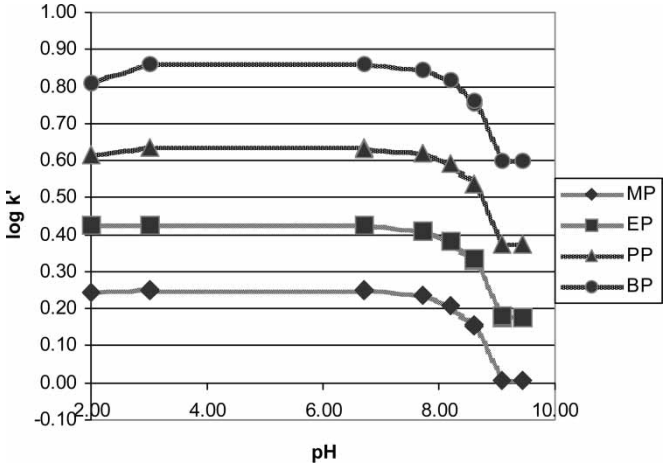

Figure 2. Curves of pH dependency of the lipophilicity parameter log k' of methylparaben (MP), ethylparaben (EP), propylparaben (PP), butylparaben (BP).

Table 1. Correlation parameters of curves of pH dependency of lipophilicity parameter k of parabens according Eq. (2)

	A	K	C	D	R^2	Obtained pKa
MP	0.2447	3.5464	0.0009	10.0923	0.996	8.87
EP	0.2518	3.6068	0.1683	10.0848	0.995	8.90
PP	0.2598	3.7263	0.3686	10.0347	0.996	8.87
BP	0.2643	3.6452	0.5913	10.0588	0.997	8.79

Eq. (4)[17] and the derived expression for pKa–Eq. (5):

$$k = \frac{k_{HA} + k_A^* 10^{pH-pKa}}{1 + 10^{pH-pKa}} \tag{4}$$

$$\text{pKa} = \text{pH} - \log \frac{k_{HA} - k}{k - k_A} \tag{5}$$

where k is the retention factor at a given pH of the compound investigated, k_{HA} and k_A are the retention factors of unionized and fully ionized forms, pKa is the pKa value of the compound investigated and pH is the pH of the environment.[17]

The lipophilicity parameter expressed by the logarithm of the retention factor k' according to the Eq. (1),[7,9,11] particularly is determined by reversed phase high performance liquid chromatography.

The partition coefficient of parabens log P was calculated using the data obtained by spectrophotometric measurement of their concentrations distribution in the n-octanol-water partition system at wavelength 254 nm, using the described below Eq. (6). The log P data were obtained at the same condition (pH value 3.0, buffer concentration 0.02 M potassium phosphate, and temperature 37°C, wavelength 254 nm) as the conditions of the chromatographic determination (Table 3).

The calculation was performed using the following equation:

$$\log P_{O/W} = \log C_O - \log C_W \tag{6}$$

Table 2. Comparison between obtained and literature pKa values

Obtained pKa values	Obtained pKa values using eq. (5)	Literature pKa values
8.87	8.95	8.47
8.90	9.16	8.50
8.87	8.87	8.47
8.79	8.94	8.47

Table 3. Log *P* determined at T°C = 37 and pH 3 with shake-flask technique experiment

Methylparaben	Ethylparaben	Propilparaben	Butylparaben
1.91 ± 0.01	2.343 ± 0.001	2.94 ± 0.01	3.50 ± 0.02

where C_O and C_W are the parabens concentrations in octanolic and aqueous phases after partitioning, respectively.

DISCUSSION

In the paraben molecules two consecutive and rival processes occur. The first one is protonation of the oxygen atom of the ester group (C═O) (first part of the curves), and the second, deprotonation of the hydroxyl group (second part of the curves). The process of protonation (relatively weak), strongly depends on the length of aliphatic chain in the ester group. That can also be seen from the curves log k'/pH at pH range lower than 3.0 and, especially, at the long chain homologues butyl and propyl-parabens (Figure 3). It is well known, that the longer is the aliphatic chain of the ester, the stronger is its positive induction effect. That increases the electron density at the oxygen of the C═O group and, therefore, stronger coordinates a proton. The process of deprotonation of the hydroxyl group is more important for the characteristics of the preservatives (lipophilicity parameters and pKa values). The inflection points in the curves, which correspond to the determined pKa values of the parabens with this method, are very close to their literature pKa values, and in excellent agreement with the values obtained with the well known Eq. (4.)

The correlation found between log *P* obtained from *n*-octanol-water partition system and log k' obtained at the HPLC conditions described above is very good to excellent ($R^2 = 0.9981$ Figure 4).

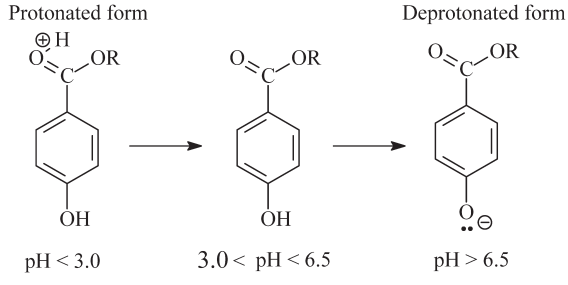

Figure 3. Protonaton and deprotonation at different pH ranges.

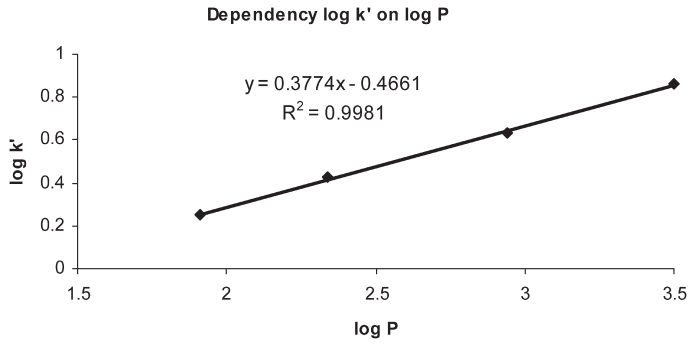

Figure 4. Correlation between log k' and log P.

Determining the best pH range of preservatives application, the pH range of their stability should be taken into account. It is known, that at pH above 8.0 a process of alkaline hydrolysis of the parabens takes place, leading to the corresponding alcohol and *p*-hydroxybenzoic acid. Above pH 7.0, considerable hydrolysis occurs.[10] This is the reason why pH ranges over 7.0 have to be avoided for parabens.

Determining the lipophilicity parameters of the parabens investigated using HPLC, and investigating the correlation between log k' and pH, the best pH ranges for preservatives applications were defined.

CONCLUSIONS

The observed correlation between pH and log k', clearly shows that a pH range optimal for each preservative exists. The best range of application for parabens is at pH values lower than their pKa value. In this range, their lipophilicity parameters have the highest values, delivering best penetration through the cellular membrane. For parabens the optimal pH is in the range of 3 to 6.5.

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