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Chromatographic behavior of zwitterionic enalapril—Exploring the conditions for lipophilicity assessment

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ABSTRACT

The chromatographic behavior of enalapril was investigated under different stationary and mobile phase conditions in an effort to unravel interferences in the underlying retention mechanism, which would affect its relation to octanol–water partitioning. Extrapolated retention factors, $\log k_w$, were used as relevant chromatographic indices. The retention/pH profile was established and the peak split phenomenon, associated with *cis*/*trans* interconversion, was also monitored as a function of pH. The pH at maximum retention and minimum peak split occurrence was chosen for further investigation, so that the presence of zwitterionic structure was guaranteed and any effect of *cis*/*trans* interconversion could be ignored. Retention of zwitterionic enalapril was found to be very sensitive to mobile phase conditions in regard to organic modifier as well to the aqueous component. The use of morpholine-propanesulfonic acid (MOPS) as buffer and the presence of *n*-octanol as mobile phase additive proved critical factors for maximum suppression of secondary interactions. Nevertheless, the corresponding extrapolated retention factor was considerably larger than octanol–water $\log D$ value at the isoelectric point. However, $\log k_w$ could be successfully converted to $\log D$ by means of a calibration equation established for ionized acidic compounds.

1. Introduction

Zwitterions constitute a category of drugs which attracts considerable interest in respect to physicochemical properties, in particular lipophilicity, expressed as *n*-octanol–water $\log D$ values, and the fraction of molecular species as a function of pH [1,2]. The interplay between partitioning, ionization state and the inter- and intramolecular interactions of the opposite charges usually leads to a bell-shaped $\log D$/pH profile. Maximum $\log D$ values are observed around the isoelectric point and are considered to be considerably lower than the theoretical $\log P$ value of the uncharged species [1,3]. Thus, despite the large arsenal of calculative approaches, lipophilicity predictions of zwitterions lack reliability and determination by direct partitioning experiments remains necessary [4]. Alternatively, extrapolated retention factors, $\log k_w$, obtained by reversed-phase HPLC under suitable conditions may satisfactorily reproduce $\log D$, while combining rapid and friendlier measurements [5–8]. In this direction, considerable research efforts have been devoted to confront secondary interactions, mainly silanophilic, which may interfere in the retention mechanism [7–9]. Silanophilic interactions depend on the presence of functional groups in the solute molecules as well as on the number, the accessibility and the ionization state of the residual silanols. Base deactivated columns possess reduced number of free silanols, while in polar-embedded columns their accessibility is restricted [10–12]. The addition of *n*-decylamine in combination with a small amount of *n*-octanol in the mobile phase has been found to produce 1:1 correlation between $\log D$ and $\log k_w$ values in the case of basic and neutral compounds, using either a conventional BDS column or a polar embedded [7,13]. For acidic compounds existing in their unionized form, addition of *n*-octanol is also successfully applied [14,15]. Lipophilic anions however show enhanced affinity for the silica-based reversed-phase columns leading to $\log D/\log k_w$ calibration equations with large negative intercepts even in presence of *n*-octanol in the mobile phase [15]. Less investigated are the chromatographic conditions for lipophilicity assessment in the case of zwitterions. The behavior of such compounds may deserve particular attention, since the stationary phase environment and/or the mobile phase composition may affect both the degree of ionization and the extent of inter- and intramolecular interactions. It should be noted that although highly polar solutes elute with the dead time in reversed-phase HPLC, not permitting determination of extrapolated retention factors [16], zwitterionic cephalosporins and fluoroquinolones have been reported to be reasonably retained

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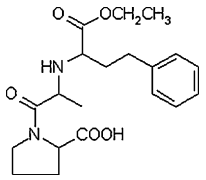

Fig. 1. Structure of enalapril.

despite their negative log *D* values. In contrast disruption of their zwitterionic structure by the addition of hydrophobic counter ions led to a remarkable decrease in retention. Moreover, a shift in the maximum of log k_w/pH profiles compared to the corresponding log *D*/pH profiles was observed [17,18].

Enalapril belongs to the angiotensin I-converting enzyme (ACE) inhibitors, occurring as a prodrug of its active metabolite the di-acid enalaprilat [19,20]. In enalapril one carboxylic group is esterified, while the second may be engaged in zwitterionic structure with the protonated basic nitrogen, depending on pH (Fig. 1). In this aspect, accurate knowledge of acidic and basic pK_a is required to assess the molecular species/pH profile. An additional feature of enalapril's physicochemical profile is its *cis*/*trans* interconversion [21]. This phenomenon is more pronounced at low temperatures and affects considerably the chromatographic behavior of enalapril leading to two peaks. Thus, usually higher temperature is suggested for enalapril analysis [22]. No systematic information is available concerning the peak split phenomenon as a function of pH or/and organic modifier concentration. To this point it should be noted that in a recent publication the lipophilicity of the two enalapril isomers has been estimated using isocratic retention factors, measured on a reversed-phase stationary phase at low temperature and pH 5.0, at which the anionic species should predominate [23]. A minor difference was found between the *cis*- and *trans*-isomer.

In the light of the above considerations we considered it interesting to focus our study on the zwitterionic enalapril and explore its chromatographic behavior under different stationary and mobile phase conditions in an effort to unravel interferences in the underlying mechanism, which would affect the analogy between reversed-phase retention and octanol–water partitioning. For this purpose the retention/pH profile was established, while the peak split phenomenon was also monitored as a function of pH. The pH at maximum retention and minimum peak split occurrence was chosen, so that the presence of zwitterionic structure was guaranteed and any effect of *cis*/*trans* interconversion could be ignored.

2. Materials and methods

2.1. Reagents

Enalapril maleate (pharmaceutical grade) was purchased by national pharmaceutical company ELPEN S.A. All reagents were of analytical grade. KH_2PO_4, $KMnO_4$ and Morpholine-propanesulfonic acid (MOPS) were supplied by Merck, Darmstadt, Germany, Na_2HPO_4 by Carlo-Erba and concentrated H_3PO_4 (85%) by Riedel de Haen. Solvents, methanol (MeOH), tetrahydrofurane (THF) and acetonitrile (ACN) were HPLC grade, purchased by Lab-Scan Science Ltd., Ireland. Octanol was extra pure purchased by Panreac Quimica, Spain. Water was deionized and further purified by means of a Milli-Q Plus water purification system, Millipore Ltd.

2.2. Potentiometry

For the determination of dissociation constants, an automatic potentiometric titrator (Shangai Rex, Shangai) was used, fitted with a glass-calomel conjugated electrode, a 10-ml automatic burette and a mechanical stirrer. 50 ml of 10^{-2} M solutions of enalapril in bi-distilled water, which had been degassed by boiling for 30 min, were titrated under nitrogen atmosphere using carbonate free KOH 0.999 M, standardized by titration with standard potassium hydrogen phthalate. Each titration was performed in triplicate. The analysis of the titration curve data was based on the average number of the unbound protons, P. Details of the method are found in Ref. [24].

2.3. Chromatographic conditions

The HPLC isocratic pumping system consisted of a GBC Model 1126 pump and a Rheodyne Model 7725i injector with a 20 μl loop, which were coupled to a GBC Model LC1210 UV–vis detector operated at 220 nm. Data acquisition was performed using WinChrom chromatography software package Version 2.1. Details are reported in Ref. [15].

2.4. Stationary phases

The following columns were used:

Hypersil BDS C-18 (250 × 4.6 mm i.d., 5 μm particle size).

Discovery end-capped BIO Wide Pore RP-18 (Supelco, USA, 150 mm × 4.6 mm i.d., 3 μm particle size).

2.5. Mobile phases

Mobile phases consisted of different organic modifier–buffer mixtures adjusted at different pH values:

With Hypersil BDS C-18 column:

(A) 20 mM KH_2PO_4 + 20–60% MeOH in the pH range 2.0–4.0, adjusted with phosphoric acid;
(B) 20 mM KH_2PO_4 + 10–20% acetonitrile, pH 3.0, adjusted with phosphoric acid;
(C) 20 mM KH_2PO_4 + 10–20% THF, pH 3.0, adjusted with phosphoric acid;
(D) 0.02 M MOPS + 20–50% MeOH, pH 3.0, adjusted with 0.1 M HCl;
(E) *n*-octanol saturated 0.02 M MOPS + 20–50% MeOH + 0.25% *n*-octanol in the volume of MeOH, pH 3.0, adjusted with 0.1 M HCl.

With Discovery end-capped BIO Wide Pore RP-18:

(F) 20 mM KH_2PO_4 + 20–60% MeOH, pH 3.0 adjusted with phosphoric acid;
(G) 0.02 M MOPS + 20–50% MeOH, pH 3.0, adjusted with phosphoric acid;
(H) *n*-octanol saturated 0.02 M MOPS + 20–50% MeOH + 0.25% *n*-octanol in the volume of MeOH, pH 3.0, adjusted with 0.1 M HCl.

Retention factors were determined at least in triplicate for each chromatographic condition and converted to log *k* values by means of the following equation:

$$\log k = \log\left(\frac{t_r - t_0}{t_0}\right) \quad (1)$$

where t_r is the retention time of enalapril and t_0 the column dead time being measured using $KMnO_4$.

2.6. Prediction of lipophilicity and ionization constants

The following software packages were used:

ClopP 4.0, based on Leo-Hansch fragmental system.

Pallas v. 3.3.2.4: the different options implemented in Prolog*P* were used: *CDR*, based on modified Rekker's fragmental system; *Atomic 6*, based on modified Ghose–Crippen atomic contribution system; *ANN2005* the recently updated Artificial Neural Network model implemented in Prolog*P*. The corresponding options of Prolog*D* were also used to calculate log *D* at the isoelectric point.

ADME.Boxes (PharmaAlgorithms, v 3.0) using Algorithm Builder (AB) which combines fragmental, descriptor and similarity based methods for log *P* prediction. log *D* at the isoelectric point was also calculated. The same software was used for pK_a prediction and their assignment to the acidic and basic group.

3. Results and discussion

3.1. Ionization constants and lipophilicity of enalapril

Acidic and basic pK_a values of enalapril calculated according to ADME Boxes are $pK_{a_1} = 3.3$ and $pK_{a_2} = 5.5$, respectively. Experimental pK_a, determined by means of pH potentiometry in the present study, were found to be $pK_1 = 2.85(\pm 0.14)$ and $pK_2 = 5.37(\pm 0.02)$. A third $pK_a = 6.54(\pm 0.02)$ determined during the titration, corresponds to the pK_a of maleic acid. According to the above results maximum lipophilicity should occur around the isoelectric pH 4.1, at which the predominant molecular species corresponds to the zwitterionic structure. This is in agreement with the log *D*/pH profile reported in literature with a maximum log *D* = −0.07 at pH 4 [25]. Among various calculation systems, predictions obtained by *C* log *P*, ADME Boxes and the Artificial Neural Network option (ANN) of Prolog*P*, are in good agreement to each other assigning log *P* = 0.89, log *P* = 0.63 and log *P* = 0.78, respectively. Other options implemented in Prolog*P* provided considerably larger log *P* values (values not given) However, there is some confusion concerning the molecular species the predicted log *P* values refer to. *C* log *P* takes into account a subtraction of −2.3 as a correction term for zwitterionic structure. ADME Boxes and ANN Prolog*P* consider the theoretically neutral form, while providing a considerably large negative value for log *D* at the isoelectric point (log *D* = −1.57 and log *D* = −1.86, respectively).

3.2. Effect of pH and organic modifier concentration in peak split

The peak split of enalapril was monitored at room temperature in the pH region 2.5–4.5 using a Hypersil BDS C-18 column with methanol as organic modifier at different concentrations. It was found to take place more often at pH > 3.0 and relatively high MeOH concentration, although its occurrence was not a strictly reproducible phenomenon. The influence of MeOH concentration in the peak split was further studied at pH 3.0 using two columns, Hypersil BDS and Discovery BIO Wide Pore C-18 columns. In both columns, peak split was more frequently observed at MeOH percentages >40%, while the non reproducible character of the phenomenon was further perceived. In Fig. 2 an example of the effect of MeOH concentration in enalapril's peak split is presented. The depicted chromatograms are obtained with the Discovery BIO Wide Pore RP-18 column using 35% (Fig. 2a) and 45% methanol (Fig. 2b). As shown, the peak split is observed only at the higher percentage of organic modifier (Fig. 2b).

3.3. Extrapolated retention factors as a function of pH

Extrapolated retention factors (log k_w) corresponding to 100% aqueous mobile phase were determined on the BDS column in the pH region 2.5–4.5 using at least four isocratic log *k* values on the linear part of the log k/φ according to the following equation:

$$\log k = -S\varphi + \log k_w \quad (2)$$

If a peak split was observed the mean log *k* value was considered. In all cases correlation coefficients $r > 0.99$ were obtained.

The effect of pH in log k_w of enalapril obtained using MeOH as organic modifier and phosphate buffer as aqueous component is presented in Fig. 3. As shown, instead of a bell-shaped log k_w/pH profile, a hyperbolic curve was obtained with an upper level plateau in the pH range 2.5–3.5. Further increase of pH was accompanied by a rapid decrease in log k_w values. To this point it should be noted that using MOPS as aqueous mobile phase component, decreased retention was observed at pH 2.5, as reported by some of us in a previous publication [15]. A possible explanation for this differentiation may be related to the zwitterionic nature of MOPS itself. As a result, MOPS does not interfere with solutes and stationary phase; thus at lower pH, protonation of the basic group becomes evident, while it may be compensated by ion pair formation in presence

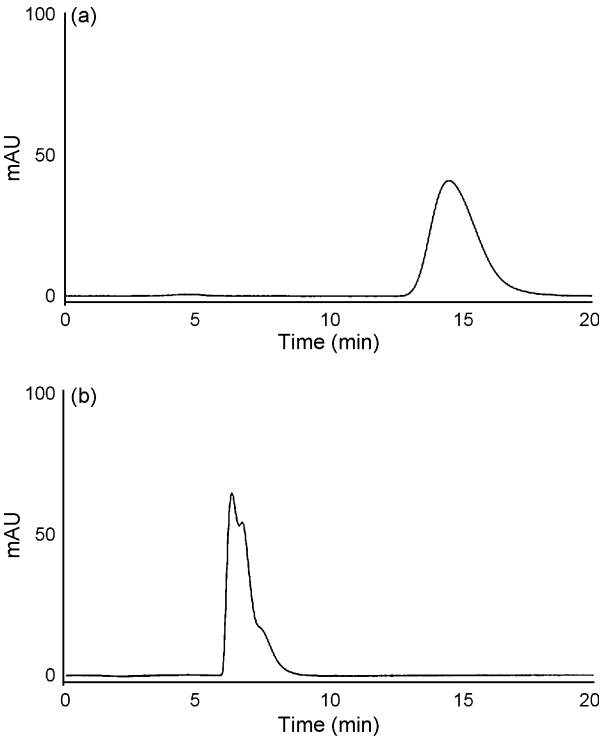

Fig. 2. Split of enalapril's peak by increasing the MeOH fraction in the mobile phase: (a) 35% MeOH and (b) 45% MeOH. (Conditions: Discovery BIO Wide Pore RP-18 column, mobile phase: 20 mM KH_2PO_4, pH 3.0, detection at 220 nm).

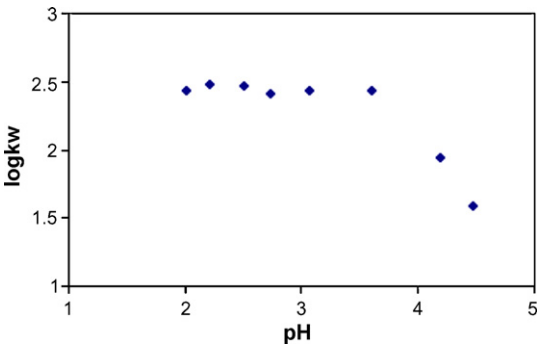

Fig. 3. Effect of mobile phase pH to extrapolated log k_w value of enalapril.

Table 1
Extrapolated log k_w values of enalapril determined under different stationary and mobile phase conditions

Mobile phase	Hypersil BDS		Discovery BIO Wide Pore C-18	
	log k_w	r^a	log k_w	r^a
KH_2PO_4/MeOH	2.40 ± 0.06	0.998	2.39 ± 0.04	0.999
KH_2PO_4/ACN	1.78 ± 0.01	0.997		
KH_2PO_4/THF	1.51 ± 0.04	0.999		
MOPS/MeOH	1.84 ± 0.017	0.9997	2.01 ± 0.04	0.999
MOPS/MeOH+ *n*-octanol	1.26 ± 0.017	0.999	1.44 ± 0.03	0.9998

[a] Correlation coefficient.

of the phosphate anions. In any case, maximum retention at pH 3.5 reveals a small shift compared to the isoelectric point and the maximum in the log *D*/pH profile at pH 4 [25]. An analogous shift to lower pH in the retention maximum has been reported in the case of zwitterionic fluoroquinolones and was attributed to the active role of the stationary phase, as well as to the fact that pH of the mobile phase corresponds to an apparent pH due to the presence of organic modifier [16].

3.4. Effect of the organic modifier to the extrapolated log k_w values of enalapril at pH 3

Considering maximum retention as well as minimization of peak split, the pH 3.0 was selected for further investigation of the chromatographic conditions most suitable for lipophilicity assessment. Extrapolated retention factors log k_w are considered as more general lipophilicity indices being in the same order as octanol–water log *D* values [5–8]. However, extrapolated retention factors may be affected by the nature of the organic modifier, as reported by a number of studies [9,26]. Such effects may be attributed to solvent selectivity as well as to changes in the stationary phase caused by the molecules of the organic modifier and the dragged water molecules during equilibration.

In the present study the influence of organic modifier on the final retention outcome was investigated by replacing methanol with acetonitrile and tetrahydrofurane. Extrapolated log k_w values were determined on the Hypersil BDS C-18 column with phosphate buffer as aqueous component, pH 3 and are presented in Table 1, along with their statistical data. Considerable differences in log k_w values, obtained with the three different organic modifiers, were observed. The decrease in polarity of the investigated organic modifiers, following the order methanol > acetonitrile > tetrahydrofurane, is reflected in the extrapolated retention factors, which increase in the same order (log k_w: 2.40 for MeOH, 1.78 for ACN and 1.51 for THF, Table 1). The log k_w produced by methanol is significantly larger than the reported experimental log *D* = −0.07 as well as the predicted log *P*/log *D* values (range: 0.63–0.89), indicating interference of secondary interactions (mainly silanophilic) in the retention mechanism. Such interactions may be partially compensated in presence of the more hydrophobic acetonitrile and tetrahydrofuran, which favour more rapid elution. Moreover, tetrahydrofuran as a stronger hydrogen bond acceptor has been postulated to drag more water molecules to the stationary phase which may act as masking agents, reducing silanophilic interactions [9].

3.5. Effect of aqueous component/stationary phase in the retention of enalapril, pH 3.0

In standard conditions reported in literature, methanol is the organic modifier of choice for lipophilicity assessment [7–9,12–15]. In this aspect, we kept methanol as organic modifier, to further investigate the effect of the aqueous component, as well as the presence of *n*-octanol as mobile phase additive, in the retention of zwitterionic enalapril on two columns Hypersil BDS and Discovery BIO Wide Pore C-18. Extrapolated retention factors, log k_w, are included in Table 1 along with their statistical data. The use of the Discovery column did not lead to significant differentiation in retention, suggesting that the end capping procedure is equally efficient and secondary interactions are practically expressed to the same extent in both columns. In contrast the mobile phase aqueous component was found to influence considerably the log k_w value of zwitterionic enalapril. Thus, the use of MOPS led to significantly lower retention on both stationary phases, compared to that observed in presence of phosphate buffer. This behavior indicates an active role of phosphate and potassium counter ions to the basic and acidic functions of enalapril. It may be suggested that, ion pair formation between opposite-charged centres may be an additional reason for enhanced retention besides the aforementioned silanophilic interactions. The addition of *n*-octanol in presence of MOPS further reduced retention on both columns as a result of its weak masking effect on the silanol sites and thereupon attenuation of silanophilic interactions. It should be noted that the decrease in retention in presence of *n*-octanol was more pronounced on the BDS column.

3.6. Comparison of extrapolated retention factors with lipophilicity

The retention of zwitterionic enalapril under the different chromatographic conditions does not reflect its hydrophilic character and indicates considerable interference of secondary interactions, silanophilic as well as ion pairing. To avoid the latter, the choice of MOPS buffer proved to be a critical factor. Addition of *n*-octanol in the mobile phase may act as a masking agent for the silanol sites, reducing retention in both columns. Nevertheless, even under conditions of maximum suppression of secondary interactions a considerable difference between log *D* and log k_w was observed. In a recent publication, some of us established a regression equation between log *D* and log k_w values for acidic compounds in their ionized form using a BDS column and methanol as organic modifier + 0.25% *n*-octanol [15].

$$\log D = 0.85 \log k_w - 0.91, \qquad (n = 21,\ r = 0.952,\ s = 0.266) \tag{3}$$

The large negative intercept in Eq. (3) reflects an enhanced retention of anionic species compared to their octanol–water partitioning. Considering that in contact with the stationary phase enalapril may behave to a certain extent rather as a carboxylate anion than as a zwitterion, Eq. (3) was applied to convert log k_w, obtained under analogous conditions, to log *D*. A value log *D* = 0.13 was calculated in good agreement with the reported experimental value at the isoelectric point (log *D* = −0.07).

4. Conclusions

The potential of reversed-phase HPLC to reproduce *n*-octanol–water partitioning in the case of zwitterionic enalapril was investigated. Retention of zwitterionic enalapril proved to be very sensitive to mobile phase conditions in regard to organic modifier as well as to aqueous component. The use of MOPS and the presence of *n*-octanol as mobile phase additive proved critical factors for maximum suppression of secondary interactions. Nevertheless, the corresponding extrapolated retention factor could be successfully converted to log *D* value by means of a calibration equation established for ionized acidic compounds, indicating that retention may be determined to a certain extent by the anionic species rather than the zwitterionic structure.

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