Physico-chemical Profiling of the ACE-inhibitor Lisinopril: Acid-base Properties
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Abstract
The acid-base chemistry of a tetraprotic, ampholyte ACE inhibitor, lisinopril, was studied by different methods. Potentiometry in aqueous medium and a co-solvent technique in methanol-water mixtures as well as 1H NMR-pH titration were applied for the highly precise measurement of protonation macroconstants. The log K values of lisinopril (at 25.0°C and 0.15 M ionic strength) were found: log K1 = 10.75 ± 0.01, log K2 = 7.13 ± 0.01, log K3 = 3.13 ± 0.01, log K4 = 1.63 ± 0.01, calculated as an average of the best two values obtained by independent methods. NMR-pH titration was used to assign the constants to the functional groups and for the examination of site-specific, submolecular basicities of the molecule (determination of protonation microconstants). In the first two well-separated protonation steps, the macro- and microconstants were identical and assigned to the primary amino group (log K1 = log kA) and to the secondary amine basicity (log K2 = log kB) respectively. The two carboxylates exhibited overlapping protonation characterised first by microconstants (log KDAB = 2.15 log kAB = 3.10), revealing that the carboxylate on the proline ring has nine times greater intrinsic basicity than the carboxylate on the side chain. The distribution of protonation species (Li2; HLis; H2Lis; H3Lis+; H4Lis2+) and microspecies (ABC; ABD) as a function of pH was calculated and used to interpret the pharmacokinetic and pharmacodynamic properties of lisinopril.

Keywords
proton speciation; logK; potentiometry; NMR-pH titration

Introduction
Angiotensin converting enzyme (ACE) inhibitors serve as fundamental medicines in the treatment of hypertension, one of the most prevalent chronic diseases nowadays. Out of the numerous currently available ACE-inhibitors, lisinopril – (2S)-1-[(2S)-6-amino-2[[1S]-1-carboxy-3-phenylpropyl]amino]-hexanoyl]-pirrole-2-carboxylic acid (Fig. 1) – belongs to the proline-containing structures.
Due to its safe and effective properties, lisinopril is widely used in the therapy of essential hypertension, symptomatic and asymptomatic left ventricular systolic dysfunction, post-myocardial infarction, renal failure and diabetic nephropathy [1]. Lisinopril is administered orally usually in a daily dose of 2.5-40 mg. Its oral bioavailability is between 25-30 % [2]. Absorption from the gastrointestinal tract does not depend on nutrition. Six hours are needed to reach the maximum plasma concentration. It hardly binds to plasma proteins. Lisinopril is not metabolised, and the absorbed drug is primarily excreted invariably in the urine [3].

A vast literature describes the pharmacology, mechanism of action, pharmacokinetics and analytics of lisinopril [3-7]. However, surprisingly little information is available on its physico-chemical properties. Lisinopril is a polyfunctional, ampholyte molecule, containing two basic and two acidic moieties. Various data have been published on its acid-base chemistry. The dissociation constants (pK\textsubscript{a} values) were reported by Bennion et al. [8] (1.68, 3.29, 7.01, 11.12) and Ip et al. [9] (2.5, 4.0, 6.7, 10.1), without an indication of the experimental error and with no assignment of basicities to moieties. Gonzalez et al. [10] assigned the measured pK\textsubscript{a} values (1.4 ± 0.1, 3.00 ± 0.01, 7.10 ± 0.01, 10.78 ± 0.01) to the functional groups, but only on the basis of chemical evidence. These three datasets of pK\textsubscript{a} values are significantly different, particularly the lowest and highest constants, which remain uncertain. The difference between them exceeds one order of magnitude.

The acid/base character determines the charge of a molecule in solution at a particular pH (characterised by the dissociation/protonation constant, pK\textsubscript{a}/log K). Further on in this paper, we use the log K term and consider the ionisation process as an association with a proton in all acid-base equilibria. This information is important in the estimation of ADME (absorption, distribution, metabolism, excretion) parameters and the interpretation of pharmacokinetic (PK) properties. Log K values can be used to better understand the binding mechanism in therapeutic events and also for optimisation of chemical reactions and analytical methods.

Several analytical methods have been applied for the determination of lisinopril in biological samples and pharmaceutical preparations such as alkalimetry [11], spectrophotometry [12], high performance liquid chromatography (HPLC) [13], high performance thin-layer chromatography (HPTLC) [14], and capillary electrophoresis (CE) [15]. Obviously, upon the application of these methods, exact knowledge of acid-base chemistry is essential (for pharmacopoeial methods) or at least favourable (for chromatographic techniques and CE).

The acidity/basicity of monovalent compounds can be quantified in terms of macroscopic log K parameters (macroconstants). For multiprotic compounds, macroconstants characterise the basicity of the molecule as a whole. They refer to the stoichiometric composition of the species, but they fail to provide information on specific proton-binding sites. Site-specific, submolecular basicities can be obtained when
microconstants are determined. Microconstants measure the proton affinity of individual groups, while the protonation states of all other sites are definite in the molecule [16]. The macroscopic and microscopic basicities provide authentic information on propensities of intermolecular interactions both in pharmacokinetic (PK) and pharmacodynamic (PD) stages [17].

As a part of our studies on the physico-chemical properties of ACE inhibitors, we investigated the acid-base chemistry of lisinopril. We characterised both the overall and the group-specific basicities of lisinopril. Validated potentiometric methods and ¹H NMR spectroscopy resulted in more precise macroconstants than in previous investigations. Here, we first studied the site-specific (microscopic) protonation equilibria of lisinopril considering the overlapping protonation of carboxylates. The results were used to interpret the PK/PD properties of the compound.

**Experimental**

**Materials and instrumentation**

Lisinopril dihydrate was generously supplied by Gedeon Richter Ltd. (Budapest, Hungary) and used without further purification. Distilled water was of pharmacopoeial grade [11] and all other reagents of analytical grade were purchased from commercial suppliers.

Potentiometric titrations were carried out on a GLpKa automated pKₐ and log P analyser (Sirius Analytical Instr. Ltd. Forest Row, UK).

NMR measurements were carried out on a 600 MHz Varian Inova spectrometer (Palo Alto, CA), equipped with a broadband inverse detection pulse field gradient probehead.

**Potentiometric logK determination**

**Electrode calibration**

The four-parameter procedure was used for electrode standardisation in both aqueous and semi-aqueous media [18]. HCl solutions of known concentration, containing 0–45.68 wt% methanol, were titrated with standardised KOH at 25.0 ± 0.1 °C, at 0.15 M ionic strength using KCl, under an N₂ atmosphere, in the pH interval of 1.8-12.2. The operational pH reading was related to p_{cH} values by the standard multiparametric equation:

\[
pH = a + S p_{cH} + j_{hi}[H^+] + j_{oh}K_w/[H^+] \tag{1}
\]

where \(a\) corresponds to the negative logarithm of the activity coefficient of \([H^+]\) at working temperature and ionic strength and \(S\) is the ratio between the electrode slope and the Nernst slope. The \(j_{hi}\) and \(j_{oh}\) terms correct the electrode junction effects at low and high pH, respectively.

The parameters were determined by a weighted non-linear least squares procedure (Refinement Pro™ 2.2 software - Sirius Analytical Instr. Ltd. Forest Row, UK).

**Titration in aqueous medium**

Six millilitres of 1.97–2.10 mM aqueous solutions of the sample were preacidified to pH 1.8 with 0.5 M HCl and then titrated alkalimetrically to pH 12. The titrations were carried out at 25.0 ± 0.1 °C, at 0.15 M ionic strength using KCl under an N₂ atmosphere. Three separate titrations were performed. The initial
estimates of \( \log K \) values were obtained from Bjerrum difference plots (\( \bar{n} \) vs. pH, where \( \bar{n} \) is the average number of bound protons) and were refined by a weighted non-linear least squares procedure using Refinement Pro\textsuperscript{TM} 2.2 software (Sirius Analytical Instr. Ltd. Forest Row, UK).

**Titration in solvent mixtures**

Three semi-aqueous solutions of lisinopril containing 14.21, 28.54 and 43.82 wt\% methanol were titrated under the same experimental conditions as in aqueous medium. The apparent protonation constants (\( \log_k \)) were calculated from the difference (Bjerrum) plot in a similar manner as the aqueous \( \log K \) values. The Yasuda-Shedlovsky procedure was applied to estimate the aqueous \( \log K \) values. The Yasuda-Shedlovsky extrapolation method is based on the linear relation between \( \log K \) and the dielectric constant (\( \varepsilon \)) of a solvent mixture:

\[
\log K + \log [\text{H}_2\text{O}] = a / \varepsilon + b
\]  

(2)

where \( a \) is the slope, \( b \) is the intercept (fitting constant) of the linear equation, \( \varepsilon \) is the dielectric constant of the methanol-water mixture and \( \log [\text{H}_2\text{O}] \) is the molar water concentration of the given solvent mixture. This method is a widely used procedure in co-solvent techniques [19,20].

**\( ^1\text{H} \) NMR titration with in situ pH monitoring**

A single NMR sample solution of 0.6 ml was prepared containing 8 mM lisinopril, 140 mM NaCl (to ensure a constant ionic strength of 0.15 M) in a 9/1 \( \text{H}_2\text{O}/\text{D}_2\text{O} \) solvent mixture. This sample also contained 2 mM of the following pH indicator molecules: dichloroacetic acid, chloroacetic acid, acetic acid, imidazole, tris(hydroxymethyl)-aminomethane (TRIS) and trimethylamine hydrochloride (TMA) in order to determine the actual pH of the sample in each titration step, according to the electrodeless single tube NMR titration method [21]. As an internal chemical shift reference, 0.5 mM of sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) was applied. The \( ^1\text{H} \) NMR spectra were recorded at 25.0 ± 0.1 °C. The water signal was suppressed either by the double pulse field gradient spin echo (dpfgse) or the selective presaturation (presat) sequence. The in situ pH value was deduced from the chemical shift of the appropriate indicator by the following equation:

\[
\text{pH} = \log K_{\text{Ind}} + \log \frac{\delta_{\text{obs}} - \delta_{\text{Hind}}}{\delta_{\text{Ind}} - \delta_{\text{obs}}}
\]  

(3)

where \( \log K_{\text{Ind}} \) is the protonation constant of the indicator and \( \delta_{\text{Ind}}, \delta_{\text{Hind}} \) are the limiting chemical shifts of its non-protonated and protonated forms (determined in separate experiments) [22,23].

The NMR-pH datasets were fitted with the Opium computer program [24] to determine protonation macroconstants and chemical shift values specific to each macrospecies (\( d\text{H,Lis} \)).

**Results and Discussion**

Among the ACE inhibitor drugs, lisinopril has the most interesting and complex acid-base chemistry. There are four proton-binding sites in the molecule: two carboxylates as well as a primary and a secondary
amine group. The protonation processes of the two carboxylate groups are expected to be highly overlapping. For the investigation of the ionisation/protonation properties of the molecule, potentiometry in aqueous medium and in methanol-water mixtures as well as NMR-pH titration were applied.

**Protonation macroconstants**

The stepwise protonation equilibria (Fig. 2) of the whole molecule are characterised in terms of protonation macroconstants, log $K$ values.

![Figure 2. Stepwise protonation equilibria of lisinopril](image)

Generally, potentiometry in aqueous medium is the method of choice for log$K$ determination for molecules with solubility greater than 0.8 mM in the entire pH interval of interest. The solubility of lisinopril (0.22 M, [9]) allows the determination of macroconstants by the standard method. The four obtained log $K$ values along with the standard deviations calculated from three parallel titrations (3 x 15 points) are listed in Table 1.

Since the log $K_4$ value falls below to the lower applicability limit (log $K < 2$) of pH-metric titration, we also measured the protonation macroconstants using the co-solvent method. The effect of methanol on the protonation constant is known to depend on the charge of the basic site. For acids, the apparent log $K$ value increases with the increasing weight percent of methanol, while a decrease is usually observed for bases [25]. Using this co-solvent method, we obtained a more reliable value for log$K_4$ since the log$K_4$ values in methanol-water mixtures shifted up to the measurable pH range. At the same time, this co-solvent method allows for assigning log$K$ values to the acidic and basic moieties of the molecule. According to the slopes of the regression lines on the Yasuda-Shedlovsky plot (Fig. 3), log $K_1$ and log $K_2$ characterise amine functions, while log $K_3$ and log $K_4$ the carboxylate groups of the molecule, respectively.

For the exact proton speciation of lisinopril, $^1$H NMR-pH titrations were carried out using the most similar possible experimental conditions as in potentiometry. Fig. 4 shows the aliphatic part of one spectrum from the titration series.
Figure 3. Yasuda-Shedlovsky plot where open diamonds denote log, $K_1$ (slope: -138.4, intercept: 14.271), open squares denote log, $K_2$ (slope: -44.6, intercept: 9.532), filled diamonds denote log, $K_3$ (slope: 82.6, intercept: 3.891) and filled squares denote log, $K_4$ (slope: 29.9, intercept: 2.976).

Figure 4. Representative $^1$H NMR spectrum of lisinopril at pH 5.07 in 9/1 H$_2$O/D$_2$O. The assignment is based on conventional 2D NMR techniques.

At certain pH values, the assignment of overlapping multiplets to methylene and methine protons was verified by TOCSY and HSQC experiments. In Fig. 5, the chemical shifts of the methine protons H(2), H(2') and H(2'') along with the methylenes H(6'), H(3'') and H(4'') are depicted as functions of pH. These six datasets were fitted simultaneously to the tetraprotic macroscopic model function described elsewhere [26]. The obtained macroconstants are shown in Table 1.
Table 1. Protonation macroconstants measured by different methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Protonation macroconstants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>log $K_1 \pm SD$</td>
</tr>
<tr>
<td>potentiometry in aqueous medium</td>
<td>10.74 ± 0.01</td>
</tr>
<tr>
<td>potentiometry in solvent mixtures</td>
<td>10.75 ± 0.01</td>
</tr>
<tr>
<td>NMR-pH titration</td>
<td>10.53 ± 0.03</td>
</tr>
<tr>
<td>average of the best two values*</td>
<td>10.75 ± 0.01</td>
</tr>
</tbody>
</table>

* see details in text

Figure 5. NMR-pH titration curves with computer fitted solid line

The macroconstants (Table 1) obtained by different methods show generally good agreement. The average deviation between the log $K$ values was 0.09 units. The ca. 0.2 unit discrepancy in log $K_1$ between data acquired by the NMR and potentiometric methods may arise from the greater ambiguity of in situ pH values. Specifically, trimethylamine has a log $K_{Ind} = 9.90$ [23] and thus can monitor pH values higher than...
10.9 with a lower precision (see the error estimations in [21] for details). In contrast, the log $K_4 = 1.63$ value by NMR can be considered as more reliable than data obtained by potentiometry in aqueous medium, because below pH 2, the acidity error of the electrode is considerable, while here the NMR-pH titration as described above is an electrodeless technique. This constant could be determined with excellent precision from the extrapolation of semi-aqueous log, $K$ values due to the reasons mentioned above.

However, we think it reasonable to make an average of the best two experimentally measured log$K$ values based on the following principle. We omit the log $K$ value with the highest uncertainty (the highest SD value). The calculated, most reliable protonation macroconstants are indicated in the last row of Table 1. Comparing the obtained log$K$ values with those reported in [9], the difference was greater than 0.4 log units in all log $K$ values. Both the log $K_1$ determined by Benion et al. [8] and the log $K_4$ reported by Gonzalez et al. [10] differ remarkably from our data. This is not surprising because those constants were deduced from conventional pH-potentiometry in aqueous medium, while the methods used in our study (potentiometry in methanol-water mixtures, $^1$H NMR-titration) give more reliable protonation constants at pH extremes.

**Protonation microconstants**

Nevertheless, macroscopic constants could not directly be assigned to functional groups, since the difference in their logarithms did not exceed 3. The microscopic protonation scheme in Fig. 6 should be considered for lisinopril instead, where the superscript on microconstant $k$ indicates the group protonating in the equilibrium in question, whereas the subscript (if any) refers to already protonated group(s).

In the first two well-separated protonation steps, the macro- and microconstants are identical. Since the first protonation is reported most sensitively by H(6'), this protonation step can be assigned to the primary amino group ($\log K_1 = \log k_A^A$). Similarly, $\log K_2 (= \log k_{A}^{B})$ accounts for the secondary amine basicity.

In the case of lisinopril, the carboxylates denoted by C and D exhibit overlapping protonation, so selective monitoring of at least one of them is a prerequisite for microconstant determination. According to the normalised NMR titration curves, H(3") and H(4") can be assumed as selective sensors of D carboxylate protonation. Thus, the experimental chemical shift profile of H(3") was fitted to the following model equation:

![Figure 6. Site-specific (microscopic) protonation scheme of lisinopril](image)

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\[ \delta^{\text{obs}} = \delta_{H_i,Ls} + (\delta_{H_i,Ls^+} - \delta_{H_i,Ls}) \cdot \frac{k_{AB}^D[H^+] + K_3K_4[H^+]^2}{1 + K_3[H^+] + K_4K_4[H^+]^2} \]  

(4)

The microscopic evaluation resulted in \( \log k_{AB}^D = 2.15 \pm 0.05 \) (n= 2) which, in turn could be converted to the remaining three microconstants, using the following Hessian constraints:

\[ k_{AB}^D + k_{AB}^C = K_3 \]  

(5)

\[ k_{AB}^Dk_{AB}^D = k_{AB}^Ck_{AB}^C = K_3K_4 \]  

(6)

We obtained \( \log k_{AB}^C = 2.63 \), \( \log k_{AB}^C = 3.10 \) and \( \log k_{ABC}^D = 1.68 \) (with estimated uncertainties of 0.05).

The main pathway of protonation includes the ABC microspecies, while its minor protonation isomer (ABD) has nine times lower abundance at all pH values. In other words, the carboxylate on the proline ring exhibits nine times greater intrinsic basicity than the carboxylate on the side chain with an adjacent, electron-attracting protonated secondary amine (-NH\textsubscript{2}+) group. The mutual basicity-modifying effect of these moieties is quantified by the interactivity parameter, defined as the difference in the corresponding microconstants: \( pE_{CD} = 2.15 - 1.68 = 3.10 - 2.63 = 0.47 \). This \( pE \) value suggests an interaction exceeding the random, Coulombic value of 0.2-0.3 \cite{27}; presumably, these sites communicate through space with a simultaneous change in their geometric positions. A conformational change at pH < 4.5 is also substantiated by the changing multiplet patterns in the NMR spectra, but a detailed analysis of vicinal \(^3 J_{HH} \) values holding conformational information is beyond the scope of this article.

**Distribution of protonation species**

Using \( \log K \) and \( \log k \) values, the percentage of various protonated species can be calculated for any arbitrary pH value. The distribution ratios of each species as a function of pH are shown in Fig. 7.

![Figure 7. Distribution curves of the macrospecies (H\textsubscript{i}Lis) and microspecies (ABC and ABD) of lisinopril](image)
Lisinopril speciation

Table 2 summarises the relative concentration of lisinopril protonation species at the most relevant pH values in the body. In the stomach, the monocation (H₃Lis⁺) and the dication (H₄Lis²⁺) forms are present at about equal levels. In the gastrointestinal tract, the double protonated H₂Lis form predominates, while at the pH of plasma, the monoanion (HLis⁻) is the dominant species.

<table>
<thead>
<tr>
<th>Form of the species</th>
<th>Percentage concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>stomach pH 1.5</td>
</tr>
<tr>
<td>Lis²⁻</td>
<td>0.00</td>
</tr>
<tr>
<td>HLis⁺</td>
<td>0.00</td>
</tr>
<tr>
<td>H₂Lis</td>
<td>0.99</td>
</tr>
<tr>
<td>H₃Lis⁺</td>
<td>42.15</td>
</tr>
<tr>
<td>H₄Lis²⁺</td>
<td>56.86</td>
</tr>
</tbody>
</table>

Conclusions

In this study, the acid-base chemistry of tetraprotic lisinopril was characterised by protonation macro- and microconstants. The highly precise log K values obtained by two independent analytical methods provided a better interpretation of the PK/PD properties. As Fig. 7 indicates, under the pH conditions of the GI tract – the most likely site of oral absorption – lisinopril is predominantly present in the H₂Lis form. Although the net charge of this species is zero, the molecule exists in solution as a double zwitterion: the two amine functions are protonated while the two carboxylates are not. This structure represents a highly polar dipole that is unfavourable for passive transport through lipid membranes, which explains the low bioavailability of the molecule. Recently, the intestinal dipeptide transporter system (DTS) has been reported to be involved in the active transport mechanism of oral absorption of ACE inhibitors [28].

At the pH of the plasma, the monoanion (HLis⁻) is the dominant form (more than 60%), which favours receptor binding. As is known, therapeutically useful ACE inhibitors exhibit three-point binding to the Zn ion-containing carboxypeptidase ACE enzyme [2]. The proline C₇-carboxylate of lisinopril binds to the positively charged Arg₁⁴₆, while the other carboxylate in the side chain participates in complex formation with the Zn ion. The third binding site is represented by Ser-OH where the C₁=O group forms an H-bond. The species distribution revealed in this study explains the good receptor binding of lisinopril.

References


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