# Proton Speciation and Microspeciation of Vinpocetine and Related Compounds in Aqueous and Biomimetic Media

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***Purpose.*** The determination of protonation macroconstants of twelve compounds in the vincamine drug family and the determination of protonation microconstants of *cis*- and *trans*-apovincaminic acid in media of various solvent composition to characterise their site-specific basicity and to estimate the concentration of the membrane-penetrating and receptor-binding forms.

***Methods.*** UV-pH titrations have been used to determine the protonation macroconstants in 10–43 wt% methanol/water mixtures. Yasuda-Shedlovsky extrapolation was applied to obtain aqueous log$K$ values for compounds sparingly soluble in water. Protonation microconstants were also determined by deductive methods for compounds of free carboxylic group.

***Results.*** In the case of the two water-soluble compounds the extrapolated and the directly measured aqueous log$K$ values were in good agreement, verifying all other extrapolated data. Compounds of *cis*-D/E ring anellation are 0.4–0.8 log$K$ units more basic than their epimeric, *trans* counterparts. The pH-dependent distribution of apovincaminic acid microspecies in aqueous and membrane-like media is depicted in microspeciation diagrams.

***Conclusions.*** The N(4) nitrogen is more shielded by the adjacent ethyl group in *trans*-D/E ring anellation eburnanes than in *cis* ones, as reflected by the protonation constants. Solvent-dependent basicity data predict superiority of *trans* isomers in lipophilicity and membrane-penetrating ability.

**KEY WORDS:** vincamine; vinpocetine; apovincaminic acid; protonation constants; microspeciation.

## INTRODUCTION

Vincamine and its semisynthetic derivatives; such as vinpocetine; are valuable cardiovascular agents in cerebral insufficiencies (1). Vinpocetine was introduced into the clinical practice two decades ago for the treatment of cerebrovascular disorders and related symptoms (2). Since then it has become a reference compound in the pharmacological research of cognitive deficits caused by hypoxia and ischaemia (3).

The neuroprotective action of vinpocetine is reported to be related to the inhibition of voltage-dependent $Na^+$-channels and indirect inhibition of some molecular cascades initiated by the rise of intracellular $Ca^{2+}$-levels (4). On the other hand, its cardiovascular effects are usually associated with the selective inhibition of the $Ca^{2+}$-calmodulin dependent, cyclic GMP specific phosphodiesterase (cGMP-PDE) enzyme (5,6). This inhibition enhances intracellular cGMP levels in the vascular smooth muscle resulting in reduced resistance of cerebral vessels and increase of cerebral flow. This effect might also beneficially contribute to the neuroprotective action (7). The medicinal use of members and derivatives (vincamine, vinpocetine, vinburnine, brovincane) of the eburnane ring system has inspired continual synthetic efforts. All these compounds are of *cis*-D/E ring anellation, i.e., 3α-H, 16α-ethyl: (3S,16S) configuration. Recently, however, an increasing attention is given to compounds with *trans*-D/E ring anellation (8,9).

Although some of these compounds have been in therapeutic use world-wide for more than 20 years, and their membrane-penetration, receptor-binding and several other biological processes greatly depend on their basicity, no previous report was devoted to their acid-base properties. In fact, the only pertinent literature data (10) refers to one single compound, *cis*-apovincaminic acid and its basicity, with no mention of the determination method. The obvious reason that prevented these compounds from acid-base characterization is their very limited water-solubility.

Here we report the determination of acid-base properties at the macroscopic level for 10 monobasic compounds, and at the macroscopic and microscopic (submolecular) levels for 2 dibasic molecules. The 12 compounds studied vary in the configuration of the C(3) carbon, saturation of the C(14)-C(15) bond, the character and chain-length of the possible substituent of the C(14)-carboxylic acid group. Eight of the compounds constitute two groups of *cis* and *trans*-D/E anellation epimeric pairs, and four of the compounds can be sorted into two groups of C(14)-configurational diastereomeric pairs. A representative set of structures can be seen in Fig. 1. The monobasic compounds are as follows: (3S,14S,16S) vincamine, (3S,14R,16S) epivincamine, (3R,14S,16S) *trans*-vincamine, (3R,14R,16S) *trans*-epivincamine, (3S,16S) ethyl *cis*-apovincaminate (vinpocetine), (3R,16S) ethyl *trans*-apovincaminate, (3R, 16S) (2-acetoxy)-ethyl *trans*-apovincaminate, (3R,16S) (2-hydroxy)-ethyl *trans*-apovincaminate, (3R,16S) (3-acetoxy)-propyl *trans*-apovincaminate, (3R,16S) (3-hydroxy)-propyl *trans*-apovincaminate. The dibasic compounds are those that contain the carboxylic acid group in its unesterified form: (3S,16S) *cis*-apovincaminic acid and (3R,16S) *trans*-apovincaminic acid.

## THEORY

Concerning general experimental techniques for the determination of protonation constants (log$K$), pH-potentiometry is certainly the most widespread, principal one. This method is fast, reproducible and inexpensive (11), but it requires relatively high solute concentration, especially when the molecule protonates near any extremum of the pH scale. If the molecule is sparingly soluble, but it possesses pH-dependent UV absorption due to a chromophore in the proximity of the protonating group, UV spectroscopy can also be a powerful tool of log$K$ determination. Molecules of strong chromophore moiety can thus be characterized in solutions of $10^{-4}$–$10^{-5}$ M concentration. Nevertheless, in our aqueous solutions, even at sample concentrations as low as $2.5 \cdot 10^{-5}$ M, precipitation occurred during the

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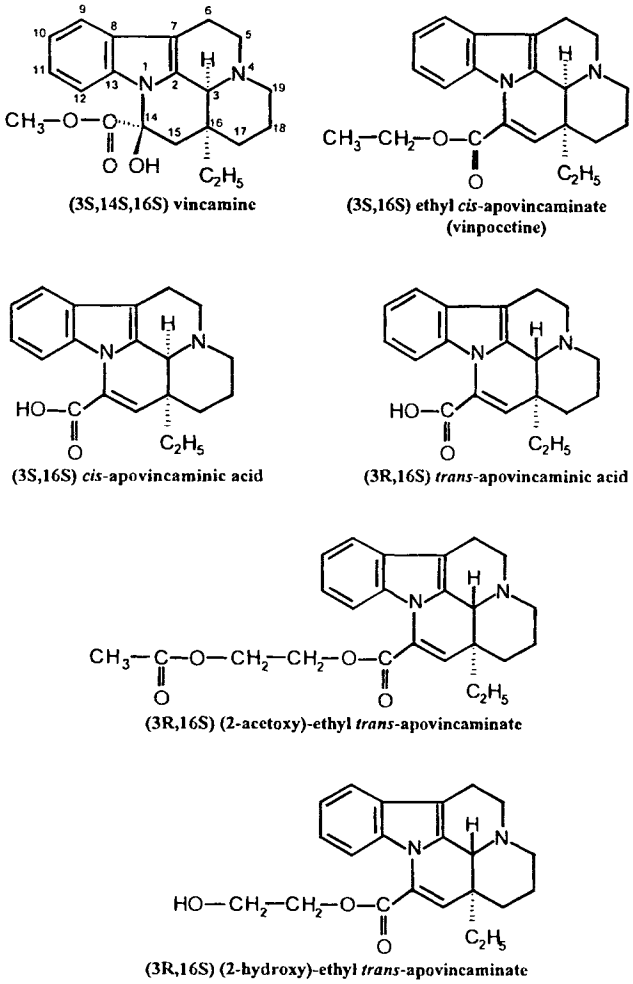


**Fig. 1.** A representative set of vincamine derivatives.

UV-pH titration, with the exception of the two apovincaminic acids.

Under conditions of very poor solubility, the feasible approach for log$K$ determination is the mixed-solvent procedure. This method is based on the determination of apparent protonation constants (log$_S K$) in organic solvent/water mixtures of various composition, where the aqueous log$K$ is obtained by subsequent extrapolation. Concerning log$_S K$ data in partly aqueous solvents, methanol is the organic component of obvious choice, since methanol shows a solvation effect closest to water. We used 3–6 different solvent mixtures, including the lowest possible methanol-containing one, which still allowed the clear dissolution of the compound studied, throughout the UV-pH titration. For extrapolation, the Yasuda-Shedlovsky procedure (12) offers benefits over the traditional plot of log$_S K$ versus wt% of methanol. This method uses a plot of log$_S K$ + log[$H_2O$] versus 100/ε, where ε is the dielectric constant of the medium. The relationship, log$_S K$ + log[$H_2O$] = 100$a$/ε + $b$, includes log[$H_2O$], the molar water concentration of the given solvent mixture, $a$ and $b$, the slope and intercept, respectively. Aqueous log$K$ values can be obtained by introducing log 55.5 and 100/78.3, the respective values of molar concentration and dielectric constant of pure water. A validation study has recently shown that errors of the Yasuda-Shedlovsky extrapolation values, in methanol wt% up to 60, are not greater than ±0.1 log$K$ units for weak bases and ±0.2 for weak acids (13). For our 12 vincamine derivatives, an even more relevant assessment of the extrapolated data was offered by the fact that *cis*- and *trans*-apovincaminic acids, as exceptions, are water-soluble. Thus their extrapolated and directly determined aqueous data could be compared.

The type of constants determined by UV-pH titrations, and obtained by extrapolation, is macroconstant, $K_1$ for monobasic compounds, and $K_1$ and $K_2$ for compounds of two basic sites. These parameters characterize the molecule as a whole, since macroconstants, in principle, can not be assigned to specific sites (14).

Basicity of the protonation sites of bidentate ligands, such as *cis*- and *trans*-apovincaminic acid, can only be correctly characterized in terms of microscopic protonation constants (microconstants) (15). Microconstants are the exact physicochemical parameters, quantitating the proton-binding capability of submolecular basic units, when the protonation states of all other sites are definite in the molecule (14,16). Also, they are the analytical tools to quantitate the concentration of the various protonation forms, of which not necessarily the major one is reactive in biological and chemical processes (17,18).

The protonation scheme of *cis*-apovincaminic acid, showing the four microspecies, their symbols, the four micro- and two macroconstants can be seen in Fig. 2. Relationships between the micro- and macroconstants have been known since Bjerrum's pioneer work (15), and can be written for apovincaminic acid as follows:

$$K_1 = k^A + k^C \quad (1)$$

$$K_1K_2 = k^A k_A^C = k^C k_C^A \quad (2)$$

where $K_1$ and $K_2$ are the stepwise macroconstants, $k^A$, $k^C$, $k_A^C$, $k_C^A$ are the microconstants, indices C and A designate the carboxylate and amino site, respectively. Superscripts of microconstants indicate the group protonating in the given microequilibrium protonation process, whereas the subscript (if any) stands for the group already holding proton during the process. $Apo^-$, HApo and $H_2Apo^+$ are macrospecies, of which HApo is a composite of two protonation isomer microspecies:

$$[HApo] = [HApo^{\pm}] + [HApo^{0}] \quad (3)$$

The protonation at one basic site modifies (usually decreases) the basicity of the other site. The measure of this basicity-modifying effect can be quantified in terms of Δlog $k$, the interactivity parameter. Its actual form for the carboxylate and amino groups can be written as:

$$\Delta\log k_{C-A} = \log k^C - \log k_A^C = \log k^A - \log k_C^A \quad (4)$$

Amino basicities typically greatly exceed those of carboxylates in amino acids and other compounds, especially in media of high dielectric constant. Accordingly, the predominant protonation of apovincaminic acids proceeds along the $k^A$ and $k_A^C$ microconstants. Nevertheless, the minor protonation isomer ($HApo^0$) does exist, and can be the reactive one in structure-controlled reactions. Furthermore, it can even be promoted to be the major species by media of low dielectric constant.

The calculation of four microconstants in a bidentate ligand needs at least three independent items of information (14). Two of them are the macroconstants, and the third one may originate

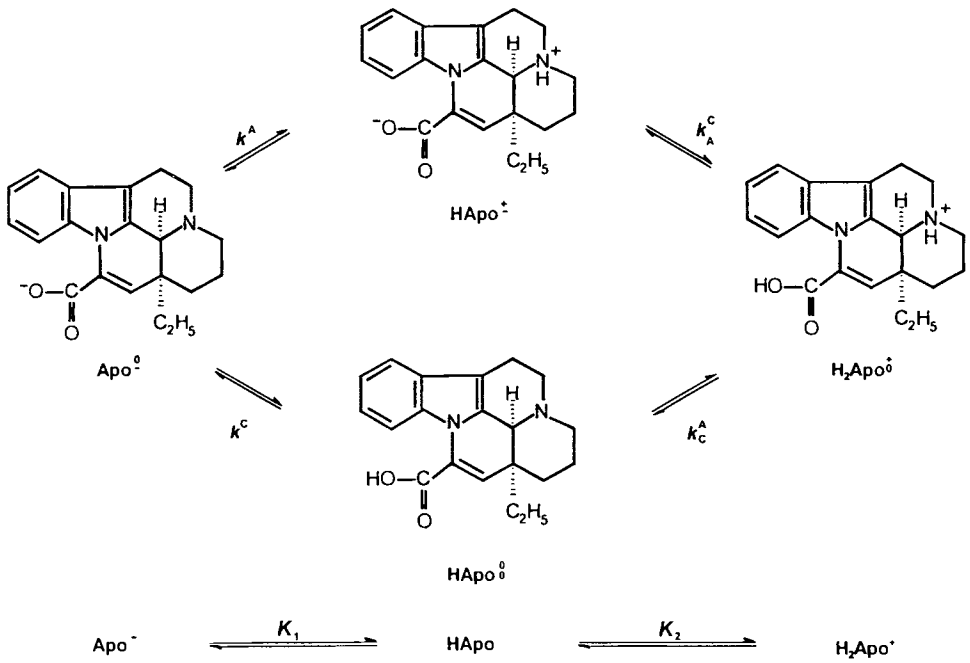


**Fig. 2.** Microscopic protonation scheme of *cis*-apovincaminic acid. Relationships between the micro- and macroconstants are defined in Eqs. (1)–(2).

from UV-pH (16), NMR-pH (19), etc. titrations, or deductively, from macroconstant(s) of related, simplified compound(s) (20). Due to the great difference between the amino and carboxylate basicity in aqueous and polar solvent solutions, the concentration of the minor microspecies ($HApo^0$) is very low, and its contribution to any spectroscopic signal is insignificant. The deductive method is therefore the only feasible one here, provided that an appropriate model compound can be found to mimic the minor protonation isomer (21,22).

It has been shown in classical works (20) and recent studies (23) that electronic (withdrawing or sending) effects of a carboxylic, –COOH group (note that definitely not a carboxylate, $-COO^-$), and a carboxylic ester, –COOR group are virtually identical on the adjoining moieties. This observation is even more plausible in apovincaminic acid derivatives where the –COOH or –COOR group is separated by several bonds from the tertiary nitrogen.

## MATERIALS AND METHODS

The twelve compounds were produced and purified by methods described earlier (8,9). Methanol was of spectroscopic grade (Chemolab), all other chemicals were of analytical reagent grade.

UV-pH titrations were carried out using a Perkin-Elmer Lambda 15 UV/VIS Spectrophotometer, a Radiometer pHC2406 combined pH electrode and a Radiometer pHM93 reference pH meter.

Each different solvent mixture needed a pH electrode standardization of its own. Slope of the pH function was based upon calibration by NBS standard buffer solutions in aqueous medium. Then the electrode was soaked for a day in the given methanol/water mixture and standardized with potassium hydrogen phthalate of molality 0.05 mol/kg in the appropriate methanol-water mixture of declared pH value in the respective solvent composition (24).

The methanol content of the solvent was 42.5, 36.0, 28.9, 20.4, 10.0 and 0.0 wt%, depending on the solubility of the compound in question, as itemized in Table I. The concentration of the solute was $7.5 \cdot 10^{-5}$ M in each titration. The $p_SH$ of a solution under UV spectroscopic investigation was adjusted by the addition of 0.1 M HCl or KOH using a Hamilton syringe. Absorption spectra of known acidity were recorded and the wavelength was chosen so that absorptions of the acidic and basic forms be as different as possible. For the determination of $K_1$ and $K_2$ macroconstants, the evaluations were made at 285 nm and 260 nm, respectively. In all UV-pH titrations 1 cm cuvettes were used. Molar absorption coefficients were typically 6480 $M^{-1}$ $cm^{-1}$ and 4650 $M^{-1}$ $cm^{-1}$ at 285 nm of the basic and N-protonated forms to follow N-protonation, and 5320 $M^{-1}$ $cm^{-1}$ and 3590 $M^{-1}$ $cm^{-1}$ at 260 nm of the basic and C-protonated forms to follow carboxylate protonation. As solubility allowed, seven of the compounds were studied in five solvent mixtures, ethyl *cis*-apovincaminate and (3-acetoxy)-propyl *trans*-apovincaminate in four mixtures, ethyl *trans*-apovincaminate in three mixtures, and the apovincaminic acids in six different media, all at room temperature (25 ± 1°C). In order to retain maximum solubility, no auxiliary electrolyte was attempted to maintain constant ionic strength. Thus, the constants are mixed ones, containing $H^+$ activities. Nevertheless, with the exception of $K_2$ of the two acids, the related ionic strength never exceeded $10^{-3}$ M, providing maximum comparability of the data.

## RESULTS AND DISCUSSION

Figure 3 contains the Yasuda-Shedlovsky plots ($(\log_S K + \log[H_2O])$ versus $100/\varepsilon$) for some of the compounds. The

**Table I.** The Protonation Macroconstants (log $K$) of the Twelve Compounds Measured in Different Methanol/Water Mixtures.

| Compound | 0.0 wt% | 10.0 wt% | 20.4 wt% | 28.9 wt% | 36.0 wt% | 42.5 wt% |
|---|---|---|---|---|---|---|
| *cis*-apovincaminic acid | 8.49 (0.04) | 8.35 (0.05) | 8.22 (0.07) | 8.15 (0.03) | 8.02 (0.06) | 7.79 (0.04) |
| | 2.30 (0.09) | 2.40 (0.08) | 2.45 (0.08) | 2.57 (0.07) | 2.76 (0.04) | 2.87 (0.05) |
| *trans*-apovincaminic acid | 7.72 (0.04) | 7.66 (0.04) | 7.54 (0.07) | 7.35 (0.03) | 7.28 (0.05) | 6.99 (0.04) |
| | 2.19 (0.06) | 2.31 (0.11) | 2.55 (0.07) | 2.75 (0.05) | 2.92 (0.06) | 3.01 (0.06) |
| ethyl *cis*-apovincaminate | — | — | 7.25 (0.04) | 7.18 (0.04) | 6.93 (0.03) | 6.66 (0.04) |
| ethyl *trans*-apovincaminate | — | — | — | 6.36 (0.04) | 6.14 (0.02) | 5.92 (0.04) |
| (2-acetoxy)-ethyl *trans*-apovincaminate | — | 6.61 (0.06) | 6.42 (0.04) | 6.36 (0.06) | 6.10 (0.03) | 6.02 (0.03) |
| (2-hydroxy)-ethyl *trans*-apovincaminate | — | 6.73 (0.05) | 6.42 (0.05) | 6.40 (0.04) | 6.24 (0.05) | 5.91 (0.04) |
| (3-acetoxy)-propyl *trans*-apovincaminate | — | — | 6.34 (0.06) | 6.29 (0.04) | 6.08 (0.04) | 5.86 (0.04) |
| (3-hydroxy)-propyl *trans*-apovincaminate | — | 6.68 (0.06) | 6.51 (0.03) | 6.43 (0.06) | 6.10 (0.04) | 5.93 (0.03) |
| vincamine | — | 7.90 (0.04) | 7.85 (0.06) | 7.72 (0.06) | 7.60 (0.03) | 7.42 (0.04) |
| epivincamine | — | 7.85 (0.05) | 7.73 (0.05) | 7.66 (0.02) | 7.55 (0.05) | 7.37 (0.06) |
| *trans*-vincamine | — | 7.24 (0.04) | 7.08 (0.04) | 6.98 (0.05) | 6.84 (0.04) | 6.62 (0.04) |
| *trans*-epivincamine | — | 7.38 (0.08) | 7.23 (0.04) | 7.07 (0.05) | 6.95 (0.04) | 6.83 (0.04) |

*Note:* The standard deviation of the constants is indicated in parenthesis.

protonation macroconstants (log$K$) of the twelve compounds in various mixed and aqueous solutions are listed in Table I. Both macroconstants of *cis*- and *trans*-apovincaminic acid could be determined directly in water as well. On the contrary, all other compounds could be investigated in partly aqueous media only, due to their limited solubility. The standard deviation of the constants is indicated in parentheses.

The only literature protonation constants, log $K_1 = 8.3$ and log $K_2 = 2.4$ are in satisfactory agreement with our corresponding data, especially when taking into account that neither temperature, nor ionic strength was indicated (10).

Parameters of the Yasuda-Shedlovsky equations and protonation macroconstants extrapolated to zero methanol content are listed in Table II. Close correlation and linearity between the $x$ and $y$ parameters of the Yasuda-Shedlovsky plot can be seen in Fig. 3 and is reflected by the standard deviations ($SD$) in Table II.

In order to assess the precision of the extrapolation procedure, directly measured and extrapolated log$K$ values of the two water soluble compounds were compared. Expressing the differences as $\Delta_1 = \log K_{1\ \text{extrapolated}} - \log K_{1\ \text{measured}}$, and analogously for log$K_2$, the following values were obtained: *cis*-apovincaminic acid $\Delta_1 = -0.01$, $\Delta_2 = -0.06$; *trans*-apovincaminic acid $\Delta_1 = +0.10$, $\Delta_2 = 0.00$.

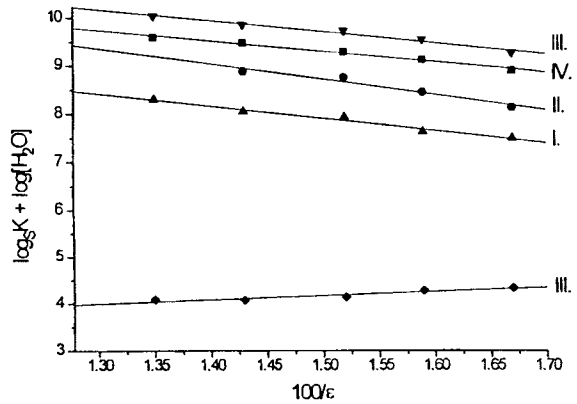


**Fig. 3.** Yasuda-Shedlovsky plots of some vincamine derivatives. ε is the dielectric constant of the mixture. I. (2-acetoxy)-ethyl *trans*-apovincaminate, II. ethyl *cis*-apovincaminate, III. *cis*-apovincaminic acid, IV. vincamine.

The plots in Fig. 3 show that the log$_S$K$_1$ values, reflecting exclusively (I, II, IV compounds) or overwhelmingly (compound III) the amino basicity are descending, whereas the log$_S$K$_2$ value (compound III), characterizing the carboxylate basicity, is ascending along with increasing methanol content, in accordance with previous findings (13,25).

Table III contains the microconstants of *cis*-apovincaminic acid and *trans*-apovincaminic acid in different methanol/water mixtures. Owing to the significantly different inherent basicities of the amino and carboxylate protonation sites, the $k^A$ and $k_A^C$ microconstants indicate the major protonation pathway and are essentially equal to the $K_1$ and $K_2$ macroconstants, respectively. Parameters of the minor protonation pathway are calculated deductively. As described above, effects of the carboxyl and ester groups on the rest of the molecule are proved to be highly similar. Thus, $k_C^A$ of the *cis*- or *trans*-apovincaminic acid and $K_1$ of the respective apovincaminic acid ester are also highly similar. Such $K_1$ values were therefore introduced into equation (2) of the pertinent apovincaminic acid, which allowed the calculation of $k^C$ as well.

A comparison of $k^A$ and $k_C^A$ as well as $k^C$ and $k_A^C$ values yielded the amino-carboxylate interactivity parameter, 0.80 for the *cis*-D/E isomer, and 0.65 for the *trans* isomer in aqueous medium. The determination of microconstants allows the construction of microspeciation tables and diagrams.

Table IV shows the occurrence probability of all the microspecies in different methanol/water mixtures at some p$_S$H values. A visual representation of the microspecies distribution of *cis*-apovincaminic acid in purely aqueous and in 42.5 wt% methanol medium can be seen in Fig. 4, where parallel curves stand for the protonation isomers. As shown, the neutral protonation isomer (HApo$^0$) gains significance at the expense of the zwitterionic protonation isomer (HApo$^{\pm}$) along with the increasing methanol content of the solvent. This microspeciation diagram is a useful tool for the prediction of isomeric

**Table II.** Parameters of the Yasuda-Shedlovsky Equations and the Protonation Macroconstants Extrapolated to Zero Methanol Content

| Compound | *a* | *b* | *r* | *SD* | *N* | Extrapolated log *K* |
|---|---|---|---|---|---|---|
| *cis*-apovincaminic acid | −2.33 | 13.20 | 0.9879 | 0,054 | 5 | 8.48 |
| | +0.87 | 2.88 | 0.9345 | 0,048 | 5 | 2.24 |
| *trans*-apovincaminic acid | −2.69 | 13.00 | 0.9908 | 0,054 | 5 | 7.82 |
| | +1.53 | 1.97 | 0.9813 | 0,044 | 5 | 2.16 |
| ethyl *cis*-apovincaminate | −3.20 | 13.52 | 0.9768 | 0,088 | 4 | 7.69 |
| ethyl *trans*-apovincaminate | −3.59 | 13.29 | 0.9993 | 0,015 | 3 | 7.07 |
| (2-acetoxy)-ethyl *trans*-apovincaminate | −2.55 | 11.74 | 0.9878 | 0,059 | 5 | 6.74 |
| (2-hydroxy)-ethyl *trans*-apovincaminate | −2.95 | 12.38 | 0.9752 | 0,098 | 5 | 6.87 |
| (3-acetoxy)-propyl *trans*-apovincaminate | −2.73 | 11.93 | 0.9768 | 0,075 | 4 | 6.70 |
| (3-hydroxy)-propyl *trans*-apovincaminate | −3.06 | 12.53 | 0.9852 | 0,078 | 5 | 6.88 |
| vincamine | −2.19 | 12.59 | 0.9919 | 0,041 | 5 | 8.05 |
| epivincamine | −2.11 | 12.39 | 0.9931 | 0,036 | 5 | 7.96 |
| *trans*-vincamine | −2.53 | 12.35 | 0.9942 | 0,040 | 5 | 7.38 |
| *trans*-epivincamine | −2.41 | 12.32 | 0.9993 | 0,013 | 5 | 7.50 |

*Note: a* is the slope, *b* is the intercept of the Yasuda-Shedlovsky equations. *r* is the regression coefficient and *SD* is the standard deviation of the fit. *N* is the number of different mixtures where investigations were made.

**Table III.** The Microconstants and the Isoelectric Point of *cis*- and *trans*-Apovincaminic Acid in Different Methanol/Water Mixtures and the Dielectric Constant of the Mixtures

| *cis*-apovincaminic acid | Aqueous | 10.0 wt% | 20.4 wt% | 28.9 wt% | 36.0 wt% | 42.5 wt% |
|---|---|---|---|---|---|---|
| log $k^A$ | 8.49 | 8.35 | 8.22 | 8.15 | 8.02 | 7.79 |
| log $k^C$ | 3.10 | 3.24 | 3.42 | 3.54 | 3.85 | 4.00 |
| log $k_A^C$ | 2.30 | 2.40 | 2.45 | 2.57 | 2.76 | 2.87 |
| log $k_C^A$ | 7.69 | 7.51 | 7.25 | 7.18 | 6.93 | 6.66 |
| isoelectric point | 5.40 | 5.38 | 5.34 | 5.36 | 5.39 | 5.33 |
| *trans*-apovincaminic acid | | | | | | |
| log $k^A$ | 7.72 | 7.66 | 7.54 | 7.35 | 7.28 | 6.99 |
| log $k^C$ | 2.84 | 3.12 | 3.46 | 3.74 | 4.06 | 4.08 |
| log $k_A^C$ | 2.19 | 2.31 | 2.55 | 2.75 | 2.92 | 3.01 |
| log $k_C^A$ | 7.07 | 6.85 | 6.63 | 6.36 | 6.14 | 5.92 |
| isoelectric point | 4.96 | 4.99 | 5.05 | 5.05 | 5.10 | 5.00 |
| dielectric constant | 78.3 | 74.3 | 69.7 | 65.9 | 62.8 | 59.9 |

**Table IV.** The Occurrence Probability of Apovincaminic Acid Microspecies in Different Methanol/Water Mixtures at Some $p_SH$ Values

| | $p_SH$ = 1.50 | | | | $p_SH$ = 7.40 | | | | $p_SH$ = 8.00 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *cis*-apovincaminic acid | $A^{\ominus}$ | $HA^{\pm}$ | $HA^{\oplus}$ | $H_2A^{\ddagger}$ | $A^{\ominus}$ | $HA^{\pm}$ | $HA^{\oplus}$ | $H_2A^{\ddagger}$ | $A^{\ominus}$ | $HA^{\pm}$ | $HA^{\oplus}$ | $H_2A^{\ddagger}$ |
| aqueous | $1.4 \cdot 10^{-8}$ | 0.1368 | $5.6 \cdot 10^{-7}$ | 0.8632 | 0.0752 | 0.9248 | $3.8 \cdot 10^{-6}$ | $7.3 \cdot 10^{-6}$ | 2.2445 | 0.7555 | $3.1 \cdot 10^{-6}$ | $1.5 \cdot 10^{-6}$ |
| 10.0 wt% | $1.6 \cdot 10^{-8}$ | 0.1118 | $8.7 \cdot 10^{-7}$ | 0.8882 | 0.1009 | 0.8991 | $7.0 \cdot 10^{-6}$ | $9.0 \cdot 10^{-6}$ | 0.3088 | 0.6912 | $5.4 \cdot 10^{-6}$ | $1.7 \cdot 10^{-6}$ |
| 20.4 wt% | $1.9 \cdot 10^{-8}$ | 0.1009 | $1.6 \cdot 10^{-6}$ | 0.8991 | 0.1315 | 0.8685 | $1.4 \cdot 10^{-5}$ | $9.7 \cdot 10^{-6}$ | 0.3760 | 0.6240 | $9.9 \cdot 10^{-6}$ | $1.8 \cdot 10^{-6}$ |
| 28.9 wt% | $1.8 \cdot 10^{-8}$ | 0.0784 | $1.9 \cdot 10^{-6}$ | 0.9216 | 0.1510 | 0.8590 | $2.1 \cdot 10^{-5}$ | $1.3 \cdot 10^{-5}$ | 0.4145 | 0.5855 | $1.4 \cdot 10^{-5}$ | $2.2 \cdot 10^{-6}$ |
| 36.0 wt% | $1.6 \cdot 10^{-8}$ | 0.0521 | $3.5 \cdot 10^{-6}$ | 0.9479 | 0.1935 | 0.8065 | $5.5 \cdot 10^{-5}$ | $1.8 \cdot 10^{-5}$ | 0.4885 | 0.5115 | $3.5 \cdot 10^{-5}$ | $2.9 \cdot 10^{-6}$ |
| 42.5 wt% | $2.1 \cdot 10^{-8}$ | 0.0409 | $6.6 \cdot 10^{-6}$ | 0.9591 | 0.2895 | 0.7105 | $1.2 \cdot 10^{-4}$ | $2.1 \cdot 10^{-5}$ | 0.6186 | 0.3814 | $6.2 \cdot 10^{-5}$ | $2.8 \cdot 10^{-6}$ |
| *trans*-apovincaminic acid | $A^{\ominus}$ | $HA^{\pm}$ | $HA^{\oplus}$ | $H_2A^{\ddagger}$ | $A^{\ominus}$ | $HA^{\pm}$ | $HA^{\oplus}$ | $H_2A^{\ddagger}$ | $A^{\ominus}$ | $HA^{\pm}$ | $HA^{\oplus}$ | $H_2A^{\ddagger}$ |
| aqueous | $1.0 \cdot 10^{-7}$ | 0.1696 | $2.2 \cdot 10^{-6}$ | 0.8304 | 0.3237 | 0.6763 | $8.9 \cdot 10^{-6}$ | $4.2 \cdot 10^{-6}$ | 0.6558 | 0.3442 | $4.5 \cdot 10^{-6}$ | $5.3 \cdot 10^{-7}$ |
| 10.0 wt% | $9.3 \cdot 10^{-8}$ | 0.1341 | $3.9 \cdot 10^{-6}$ | 0.8659 | 0.3547 | 0.6454 | $1.9 \cdot 10^{-5}$ | $5.2 \cdot 10^{-6}$ | 0.6863 | 0.3137 | $9.0 \cdot 10^{-6}$ | $6.4 \cdot 10^{-7}$ |
| 20.4 wt% | $7.5 \cdot 10^{-8}$ | 0.0818 | $6.8 \cdot 10^{-6}$ | 0.9182 | 0.4201 | 0.5799 | $4.8 \cdot 10^{-5}$ | $8.2 \cdot 10^{-6}$ | 0.7425 | 0.2575 | $2.1 \cdot 10^{-5}$ | $9.1 \cdot 10^{-7}$ |
| 28.9 wt% | $7.5 \cdot 10^{-8}$ | 0.0532 | $1.3 \cdot 10^{-5}$ | 0.9468 | 0.5287 | 0.4712 | $1.2 \cdot 10^{-4}$ | $1.1 \cdot 10^{-5}$ | 0.8171 | 0.1829 | $4.5 \cdot 10^{-5}$ | $1.0 \cdot 10^{-6}$ |
| 36.0 wt% | $6.1 \cdot 10^{-8}$ | 0.0366 | $2.2 \cdot 10^{-5}$ | 0.9634 | 0.5686 | 0.4314 | $2.6 \cdot 10^{-4}$ | $1.4 \cdot 10^{-5}$ | 0.8400 | 0.1601 | $9.6 \cdot 10^{-5}$ | $1.3 \cdot 10^{-6}$ |
| 42.5 wt% | $9.7 \cdot 10^{-8}$ | 0.0300 | $3.7 \cdot 10^{-5}$ | 0.9700 | 0.7199 | 0.2801 | $3.4 \cdot 10^{-4}$ | $1.1 \cdot 10^{-5}$ | 0.9110 | 0.0890 | $1.1 \cdot 10^{-4}$ | $9.1 \cdot 10^{-7}$ |

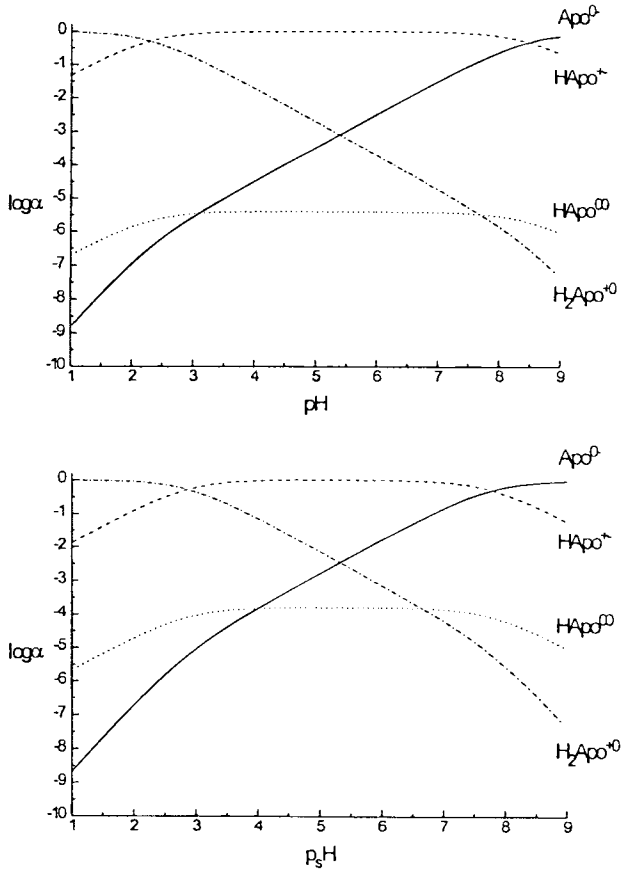


**Fig. 4.** The microspeciation of *cis*-apovincaminic acid in aqueous medium and in a mixture of 42.5 wt% methanol. α is the occurance probability of the relevant microspecies.

binding propensity in site-specific interactions with other biomolecules, where apovincaminic acid can take part as mono- or divalent proton-donor or acceptor agent.

Conclusions that can be drawn from the protonation constants are as follows:

1. The $K_1$ macroconstant (representing in highly polar media mainly the amino group protonation) of molecules with *cis*-D/E ring anellation is always higher than that of its *trans*-D/E ring anellation epimeric counterpart. This difference is 0.77 in the case of the two apovincaminic acids, while it is 0.62 in the case of their ethyl ester derivatives. Concerning the vincamine- and epivincamine-derivatives, the difference is 0.67 and 0.46 respectively. This difference is systematic with no exception and can be traced back to the different anellation, which can further be divided into skeletal, nitrogen-(4) lone electron-pair orientational and C(16)-ethyl conformational reasons. Some earlier, related observations showed significant differences between the *cis* and *trans* isomers by other techniques as well. It was reported (8) that 3,16-*trans* and 3,16-*cis* compounds of eburnane skeleton exhibit characteristic differences in their $^1$H- and $^{13}$C-NMR spectra, e.g., 3-H is about 0.9 ppm more shielded in the *trans* isomer which is due to the gauche-positioned lone electron pair of N-4 and the anisotropic shielding of the D ring. Epimeric eburnane structures undergo different fragmentation, as shown by characteristic mass spectral differences (26). The formation of $[M-70]^+$ ions shows high stereospecificity and is characteristic of the *cis* epimers, whereas these fragments are virtually absent in the mass spectra of *trans* isomers. In addition to these physicochemical parameters, *cis* and *trans* epimers of vinca alkaloids show remarkably different biological activity. It has been reported that *trans* isomers, regardless of the configurations of the two chiral centres, have two to four times higher affinities to human serum albumin than the corresponding *cis* isomers (27).

On the other hand, the anellation-dependent different rotation of the C(16)-ethyl group may well also contribute to this significantly different basicity, which is scarcely known among compounds exhibiting only stereochemical differences (14,28). Molecular modeling shows that the ethyl group in the *trans* isomer can approach much more closely the basic N(4) nitrogen than in the *cis* isomer. Thus, it can enrich the local environment of the basic site in the organic component of the solvent mixture (increasing the local hydrophobicity), and can repel the hydrated proton. Accordingly, protonation of the amino group of the *trans*-derivative occurs at higher bulk hydrogen-ion concentration (lower pH). In fact, it was reported earlier that alkyl substituents in ring systems can lower basic strength by hindering the protonation process (29), despite the fact that they actually increase the electron density of the basic site(s). Last, but not least the different solvation was also manifested by the bare fact that ethyl-*cis*-apovincaminate was sufficiently soluble in 20.4 wt% methanol to carry out protonation studies (Table I), while the *trans* isomer was not, indicating that the preferential hydrophobic solvation is an expressed property of the *trans* epimer.

2. The difference in N-basicity of apovincaminic acids and their ester derivatives can readily be interpreted. In the pH range of the amino protonation, the carboxyl groups of the acids are predominantly deprotonated, thus negatively charged, at least in media of high dielectric constant. Hence they do not have a strong electron-withdrawing effect, unlike the uncharged ester groups in the respective ester derivatives.

3. The difference between the amino and carboxylate basicities gradually decreases upon increasing the methanol content of the solvent. Based on the Yasuda-Shedlowsky equations; it is possible to estimate the methanol content and dielectric constant where the concentration of the apovincaminic acid protonation isomers are identical. For *cis*-apovincaminic acid, this value is approximately 38 which corresponds to a 92 wt% methanol solution, whereas in the case of *trans*-apovincaminic acid this value is near 45, corresponding to a 76 wt% solution. If the dielectric constant of the medium is even smaller, the neutral protonation isomer ($HApo^0$) can predominate over the zwitterionic ($HApo^{\pm}$) form at any pH. The dielectric constant-dependent constants allow estimation for the ratio of the protonation isomers. Considering a medium of $\varepsilon = 4$ (a reported dielectric constant on receptor surfaces (30), which may also well be the case in some locations inside a cell membrane lipid bilayer), the ratio between the neutral protonation isomer and the zwitterionic protonation isomer can be approximated by $2 \cdot 10^1$ for *cis*-apovincaminic acid, whereas the analogous value is $3 \cdot 10^3$ for *trans*-apovincaminic acid. Thus it can be predicted that the membrane permeability of the *trans* epimer greatly exceeds that of the *cis* epimer.

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