Determination of physico-chemical parameters influencing the analytical and therapeutic properties of vinpocetine and related compounds

PhD Dissertation Theses

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1. Introduction

Detailed knowledge of the acid-base properties and lipophilicity of drug molecules is essential in unravelling their fate in the human body (solubility, membrane penetration, binding to plasma proteins and receptors, metabolism), their analytical determination and in constructing structure-activity relationships. Although vincamine, vinpocetine and its derivatives have been in therapeutic use worldwide for several decades, very few reports appeared on their acid-base properties and lipophilicity. Some of the 12 compounds studied (Fig.1.) have therapeutic activity or are the metabolites of drugs, others are potential drug candidate molecules. Eight of the compounds constitute two groups of \textit{cis} and \textit{trans}-D/E anellation epimeric pairs, and four of the compounds can be sorted into two groups of C(14)-configurational diastereomeric pairs. There are 2 carboxylic acids and 10 esters among the molecules. The esters vary in the character and chain-length of the substituent of the C(14)-carboxylic acid group. The 4 vincaminic acid derivatives are methyl esters (vincamine and its diastereomers), among the 6 apovincaminic acid derivatives there are 2 ethyl (vinpocetine and its epimer), 2 hydroxy-alkyl and 2 acetoxy-alkyl esters, respectively.

Here we report the determination of acid-base properties at the macroscopic level ($K$) for the 10 monobasic compounds, and at the microscopic (submolecular) level ($k$) for the 2 dibasic molecules.

Knowledge of the protonation constants is also important in planning analytical methods, like capillary electrophoresis that is developing with the highest speed among separation techniques. In this technique the basis of
Figure 1. Structure of the 4 vincamine and 8 vinpocetine derivatives and the determined $\log K$ and $\log P_{TLC}$ values.
separation is the mobility difference of the analytes, which is proportional to their pH-dependent charge difference that can be calculated in the knowledge of the protonation constant. Here we report the HPCE separation of these molecules and the influence of changing parameters of the background electrolyte on the separation of these compounds. As a theoretical background we examined the applicability of Offord’s equation to partly non-aqueous media.

Due to the poor water solubility of the esters their lipophilicity could only be characterized by reversed-phase thin-layer chromatography. Here we report the octanol-water partition coefficient ($P$) of these molecules and structure-lipophilicity relationships.

2. Methods

2.1. Determination of protonation constants by UV-pH titrations

Because of the poor alkaline solubility of the esters UV-pH titrations in different water-methanol mixtures were carried out to determine their apparent protonation constants that were subsequently extrapolated to aqueous ones. 3 to 6 mixtures with methanol content between 42.5 and 0.0 wt% were used, beginning with the lowest possible methanol content that assured no precipitation during titration. A Perkin-Elmer Lambda 15 UV/VIS Spectrophotometer was used to record the spectra. 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 in the appropriate methanol-water mixture of declared pH value.
2.2. **Capillary electrophoresis separation**

The stock solution containing all of the compounds was prepared in methanol. Separations were carried out using a Crystal CE 300 instrument, equipped with a UV detector. The detection wavelength was 200 nm. A fused-silica capillary of 75 µm inner diameter and a constant voltage of 30 kV was used.

2.3. **Determination of the partition coefficients by thin-layer chromatography**

The simultaneous determination of retention values in RP-TLC assures the comparability of the calculated lipophilicity values. The calibration set included five pyrido[1,2-a]pyrimidines and the lipophilic progesterone and chlorpromazine. The accuracy of the method was tested by stir-flask logP determination on compounds with sufficient water-solubility.

Plates pre-coated with layers of silanized silica gel were used. Before applying the spots the plates were dipped into a solution of NaOH to deprotonate the residual silanol groups. The compounds were spotted on the plate from a stock solution in methanol. Developments were carried out with 7 mixtures of varying acetone and 0.1 M NaOH content. $R_M$ values were extrapolated to $R_{M0}$ ones (corresponding to an eluent of no organic co-solvent). The acetone content of the mobile phase was in the 35 to 65 V/V% concentration range with 5% increments. After development the plates were dried and the spots were detected by densitometry.

2.4. **Determination of the partition coefficients by the stir-flask method**
In the determination of logD values the use of the same buffer on different pH values is essential. By using citric acid, phosphoric acid and lysine we could produce buffers with high buffer capacity in a broad pH range (pH=2-12). After collecting samples from the aqueous phase for the subsequent UV-absorbance measurements, water-saturated octanol was added. The phase ratios were chosen in a way to assure that after partition an analytically measurable amount of the analyte remain in the aqueous phase. The two phases were intensively stirred for 2 hours in thermostated glass cells at constant temperature (25±0.1 °C). After separation of the equilibrated phases in a centrifuge the concentration of the solute was determined in the aqueous phase by UV spectrophotometry.

3. New scientific results

3.1. Determination of protonation constants

The protonation constants of the 12 molecules were determined in 5 different methanol-water mixtures and in the case of apovincaminic acids also in an aqueous solution. Among the extrapolation methods the Yasuda-Shedlovsky method was chosen. The resulting aqueous protonation constants can be seen in Fig. 1. In order to assess the precision of the extrapolation procedure, directly measured and extrapolated logK values of the two water soluble compounds were compared. The differences did not exceed 0,10 logK units proving the validity of the extrapolation method.

3.2. The calculation of protonation microconstants
Since the concentration of the minor microspecies is very low, its contribution to any spectroscopic signal is insignificant. Thus we applied the deductive method using the carboxylic acid esters. If we assume that the macroconstants of the ethyl esters are essentially the same as the $k_{o}^{N}$ microconstants of the appropriate apovincaminic acids, all of the microconstants can be calculated as can be seen in Fig. 2.

We determined all of the microconstants of both apovincaminic acids in each of the 5 methanol-water mixtures and in pure water as well. In the knowledge of the microconstants the pH-dependent distribution of the microscopic forms of apovincaminic acid molecules could be represented both in tables and plots (Fig.3.).

Fig.3. shows that the pH-independent concentration ratio of the protonation isomers depends on the methanol content, thus the dielectric constant of the mixture. The noncharged $HA_{0}^{0}$ protonation isomer gains significance at the expense of the zwitterionic $HA^{+}$ protonation isomer along with the increasing methanol content of the solvent.

3.3. **Discussion of protonation macro- and microconstants**

Protonation constants of the amino group of molecules with *cis*-D/E ring anellation are higher by 0.46 to 0.77 log units than those of their *trans*-D/E ring anellation epimeric counterparts. This difference can be traced back for one part to the different anellation influencing the electron density around the basic N(4) atom. On the other hand, orientation of the C(16) ethyl group is dependent on the D/E ring anellation. Namely, during rotation the ethyl group in the trans epimers can approach more closely the basic N(4) nitrogen than in the case of the cis epimers. Molecular mechanics
Figure 2. Microspeciation scheme of cis-apovincaminic acid and logarithms of the aqueous protonation constants

Figure 3. The pH-dependent distribution of the microspecies of cis-apovincaminic acid in aqueous solution and in a mixture containing 42.5 wt% methanol. $\alpha$ is the occurrence probability of the microspecies
calculations show that the methylene carbon of the ethyl group is closer to the N(4) nitrogen by 0.148 Å in trans epimers than in cis ones. Thus in the trans epimer the rotation of the ethyl group increases 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).

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. Hence they do not have a strong electron-withdrawing effect, unlike the uncharged ester groups in the respective ester derivatives.

The difference between the amino and carboxylate basicities gradually decreases upon increasing the methanol content of the solvent. If parameters of the Yasuda-Shedlowsky equations are still valid in media even richer in methanol, in a medium of \( \varepsilon = 4 \) (a reported dielectric constant on receptor surfaces, which may also well be the case in some locations inside a cell membrane lipid bilayer), the ratio between the noncharged and zwitterionic protonation isomers can be approximated by 20 for the cis epimer, whereas the analogous value is 3000 for the trans epimer. Thus it can be predicted that the membrane permeability and the octanol-water partition coefficient of the trans epimer exceeds that of the cis epimer. The experimentally determined partition coefficients later proved this assumption.

### 3.4. Capillary electrophoresis separation
When planning the separation we constructed the diagram showing the pH-dependence of the relative mobility of the 12 compounds based on Offord’s equation. The optimum pH range for the separation of these compounds is 7.0 to 7.5. But molecules with similar basicity and molar mass have nearly identical relative mobilities so their separation with zone electrophoresis is not possible. Therefore we chose 7 molecules out of the 12 that were especially interesting (they are in therapeutic use, drug candidates, drug metabolites) and their relative mobilities showed sufficient differences in the above pH range.

Separations (Fig.4.) were completed in the pH 7.0 to 7.5 range with buffers containing MES and TRIS, the experimental conditions were varied in 7 different running buffers based on triangular resolution mapping. We showed how changes in the parameters of the running buffer influenced the resolution of adjacent peak pairs. The three experimental variables were as follows: the total concentration of the buffer (thus its ionic strength), the ratio of the buffer components (determining the $p_S$H of the solution), the percentage of organic modifier (methanol) added. There were 3 reasons for the addition of methanol: to examine whether our previously measured $\log_SK$ values can be used for the prediction of migration order in partly non-aqueous medium as well, whether the addition of methanol increases the resolution between adjacent peaks, and methanol was also used as EOF marker.
Figure 4. The separation of 7 compounds: 26 mM MES-24 mM TRIS (pH=7.00); capillary: fused-silica, voltage: 29.8 kV; electric current: 21.2 µA. Compound assignments: vincamine (1), ethyl cis-apovincamate (2), trans-vincamine (3), (2-hydroxy)-ethyl trans-apovincamate (4), (2-acetoxy)-ethyl trans apovincamate (5), cis-apovincaminic acid (6), trans-apovincaminic acid (7).

The predicted migration order was in flawless agreement with the experimental one in each buffer. We proved the validity of Offord’s equation for the 7 alkaloids, not only in aqueous, but in partly non-aqueous solutions as well. Since the average charge is a function of the protonation constants, the in-capillary validity of these constants in partly non-aqueous media is also justified. Thus, partly non-aqueous protonation constants and the derived theoretical mobilities are able to predict experimental electrophoretic mobilities.

3.5. Determination of partition coefficients by thin-layer chromatography
The $R_M$ values of the 17 compounds decreased gradually with increasing acetone content of the mobile phase, the $R_{M0}$ values were determined by linear extrapolation. The following calibration equation was obtained using the five pyrido[1,2-a]pyrimidines in the calibration set:

$$\log P = 1.157 R_{M0} + 0.4223 \quad s = 0.07285 \quad r = 0.9951$$

The standard error and correlation coefficient confirm that the separation mechanism is partition of the analyte between the mobile and stationary phases.

The $\log P_{TLC}$ and $\log P_{shake-flask}$ values showed an excellent agreement for chlorpromazine ($\log P_{TLC} = 5.07$, $\log P_{shake-flask} = 5.13$) and a sufficiently good one for progesterone ($\log P_{TLC} = 3.75$, $\log P_{shake-flask} = 3.54$). These compounds were added therefore to the calibration set. The unified calibration equation is as follows.

$$\log P = 1.147 R_{M0} + 0.4188 \quad s = 0.1102 \quad r = 0.9972$$

The above equation was used for the determination of $\log P_{TLC}$ values of the eburnane alkaloids (Fig.1.).

### 3.6. Determination of partition coefficients by the stir-flask method

Reliability of the measured $\log P_{TLC}$ values is further justified by the $\log P$ values of vinpocetine, trans-vinpocetine and 3 vincamine epimers that could be examined by the stir-flask method. Due to the limited solubility of their neutral form, distribution coefficients could be determined in acidic solutions only, where the concentration of the neutral form is below that of the cationic one. Nevertheless, in the knowledge of the protonation constant the partition coefficients for both the neutral ($P_N$) and the cationic ($P_C$) forms could be calculated. Since partition of the cationic forms depends on the ionic environment in the solution, the calculated $P_C$ values are constants. 

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under conditions of a given buffer composition. At each pH value, besides the measured log\(D\) values, the relative concentration of the neutral form in the aqueous phase and its relative contribution to the apparent partition coefficient are also listed. Partition coefficients of the neutral form exceed that of the cationic one by at least four orders of magnitude in each case. The apparent partition coefficients of cis- and trans-apovincaminic acids were also determined on several pH values, including alkaline solutions, since with these compounds we encountered no solubility problems.

We compared the log\(P\) values of the five alkaloids where both stir-flask and RP-TLC determinations were carried out and the average deviation was 3.4%, a fairly small value in light of the reported, typical 5-10% methodical disagreement. Thus, the results of the stir-flask method justify the use of the RP-TLC method. Therefore, conclusions on structure-lipophilicity relationships were drawn from the complete set of log\(P_{\text{TLC}}\) values.

### 3.7. Discussion of partition coefficients

The alkaloids were found to have lipophilicity values in the 2.94 to 4.80 log\(P_{\text{TLC}}\) range. The log\(P\) differences reflect either electronegativity changes in the different molecular constitutions, or modulatory effects because of structural isomerism.

The apovincaminic acid derivatives are more lipophilic than the vincaminic ones, caused obviously by the absence of the polar hydroxyl group in position C(14).

The chain length and the character of the substituent of the C(14)-carboxylic acid group also influences the lipophilicity. The addition of a methylene group reduces the side chain polarity, increasing thus the lipophilicity. The terminating functional group of the side chain makes little
difference in this phenomenon. In the case of the acetoxy-derivatives the difference between the $\log P_{\text{TLC}}$ of the propyl and ethyl side chain is 0.22 log units, whereas in the hydroxy-derivatives it is 0.27 log units. Hydroxyl groups are more polar than ester groups, the difference is 0.88 log units in the propyl and 0.83 log units in the ethyl derivatives, respectively.

The $\log P$ values of the three pairs of epimers that differ in the configuration at C(3) can be interpreted as follows. There is only a negligible lipophilicity difference between the two vincamine epimers, but the trans epimers of vinpocetine and epivincamine are more lipophilic than the ones with cis anellation, showing the respective 0.57 and 0.35 log units differences. There are two major factors that contribute to the higher lipophilicity of the trans epimers. First, ring D in cis epimers occupies a perpendicular position to the plane of the ring system, whereas it is parallel in the trans epimers. Thus the smaller hydrophobic surface area of cis epimers makes them less lipophilic. The other reason is the anellation-dependent orientation of the C(16) ethyl group that was already discussed when we interpreted the protonation constants. The ethyl group can hamper the water accessibility of N(4) in the trans epimers to a greater extent and renders them more lipophilic.

4. Applicability of the results

Our basic research on the basicity and lipophilicity of these molecules contributes to a better understanding of their fate in the human body. We showed how the basicity of individual functional groups, thus the charge of the molecule, its receptor binding and membrane penetration changes in biomimetic media with lower dielectric constant. Since the specific
interactions of biomolecules are realized in their appropriate microscopic forms (in terms of protonation and conformation) and in biochemical reactions not always the dominant form is the active one, we determined the relative concentrations of all the microspecies in each of the examined media. The *in vitro* results, adapted to the *in vivo* circumstances of the compartments of the human body, can contribute to understanding and therapeutically influencing biological processes at the molecular level.

The incorporation of these log$K$ and log$P$ values into structure-activity relationships provides the opportunity of developing new, more potent drug molecules.

5. Scientific publications connected to the dissertation

Noszál Béla, Mazák Károly, Szakács Zoltán: Gyógyszervegyületek mikroegyensúlyai.  

Károly Mazák, András Nemes, Béla Noszál: Proton speciation and microspeciation of vinpocetine and related compounds in aqueous and biomimetic media.  
*Pharmaceutical Research*, 16 (11), 1757-1763 (1999)  I.F.: 2.847

Károly Mazák, Zoltán Szakács, András Nemes, Béla Noszál: Capillary electrophoresis separation of vinpocetine and related compounds: Prediction of electrophoretic mobilities in partly aqueous media.  

Károly Mazák, József Vámos, András Nemes, Ákos Rácz, Béla Noszál: Lipophilicity of vinpocetine and related compounds characterized by reversed-phase thin-layer chromatography.  
6. Conference lectures and posters

Mazák Károly: Apovinkaminsav-származékok sav-bázis tulajdonságainak jellemzése.

SOTE Gyógyszerészi Kémiai Intézet alapításának 50. évfordulója, Budapest, 1999 jún. 17.

Mazák Károly, Szakács Zoltán, Noszál Béla: Vinpocetin és rokon vegyületek speciációja és mikrospeciációja, elektroforetikus elválasztásuk predikciója és jellemzése vizes és biomimetikus közegben.
Congressus Pharmaceuticus Hungaricus XI., Siófok, 1999 okt. 6-10.

Mazák Károly, Nemes András, Szakács Zoltán, Noszál Béla: Vinpocetin és rokon vegyületei kapilláris elektroforézis elválasztása és elektroforetikus mobilitásuk predikciója.
Fiatal Kémikusok Előadóülése, Budapest, 1999 nov. 23.

Károly Mazák, András Nemes and Béla Noszál: Proton Speciation and Microspeciation of Vinpocetine and Related Compounds in Aqueous and Biomimetic Media
EUFEPS 2000, Budapest, szeptember 16-19, 2000

Károly Mazák, Zoltán Szakács, András Nemes and Béla Noszál: Capillary Electrophoresis Separation of Vinpocetine and Related Compounds. Prediction of Electrophoretic Mobilities in Partly Aqueous Media
EUFEPS 2000, Budapest, szeptember 16-19, 2000

Mazák Károly, Nemes András, Szakács Zoltán, Noszál Béla: Elektroforetikus mobilitás predikció a nemvizes közegű kapilláris elektroforézisben.

Congressus Pharmaceuticus Hungaricus XII., Budapest, 2003 máj. 8-10.