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PAPER

Dedicated to Professor Mihaela Hillebrand
on the occasion of her 85th anniversary

THERMODYNAMIC STUDY FOR THE SECONDARY EQUILIBRIA INVOLVING SILDENAFIL, VARDENAFIL AND TADALAFIL DURING ELUTION PROCESS IN REVERSED-PHASE HPLC

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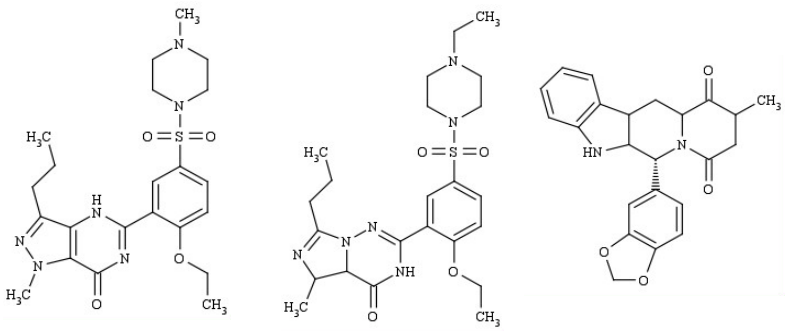
The influence of pH on retention in the HPLC process is evaluated in this study for three chemically related compounds: sildenafil, vardenafil, and tadalafil. The equilibria taking place in mobile phase have consequences on the thermodynamic behavior under reversed-phase conditions, using C8 and C18 as stationary phases. Two organic modifiers were individually used: methanol and acetonitrile. Linear and polynomial regressions were applied to the van't Hoff plots at various pH values of the aqueous component of the mobile phase composition.



Deviations from linearity were explained by the multiple forms of the studied compounds that result from different interactions in the mobile and stationary phases. Sildenafil and vardenafil being involved in several secondary equilibria that take place in mobile phase under the influence of pH have shown deviations from linearity, with two distinct domains for the van't Hoff plots: a temperature domain where the retention is controlled by enthalpy and another domain that is apparently controlled by entropy. Tadalafil being present in mobile phase in only one structural form showed a temperature behavior obeying in almost all situations to the linear van't Hoff dependence.

INTRODUCTION

The three studied pharmaceutical compounds are known for correcting erectile dysfunction with different effects by inhibiting phosphodiesterase type 5 (PDE5) inhibitors.[1] Their structures are relatively complex (as indicated in Fig. 1). They have been studied for analytical purposes, for example in the view of determining in various natural dietary supplements by means of gas-chromatography[2] or liquid chromatography.[3,4]

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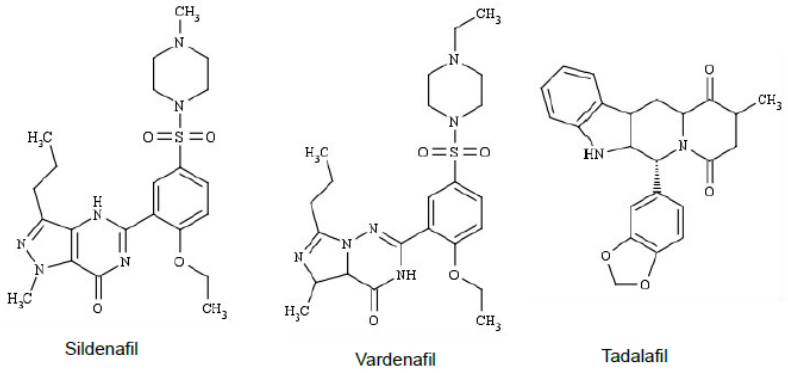

Fig. 1 – Chemical formulas for sildenafil, vardenafil and tadalafil.

Sildenafil and vardenafil are structurally similar, containing a core pyrazolopyrimidinone / imidazotriazinone ring system, while tadalafil differs significantly from the previous two compounds, possessing a tetracyclic, fused-ring structure, specifically a piperazino[1',2':1]pyrido[4-b]indole-1,4-dione system.[5] The structural differences between these compounds may result in different interactions with PDE5 and, consequently, different inhibiting effects.[6,7] Such structural differences are likely to also affect the HPLC elution process under the influence of the mobile phase composition. The main parameter that can affect the molecular structure of the studied solutes is pH of the aqueous component, which was controlled using a phosphate buffer as described in the Experimental section.

Thermodynamic investigation of the retention process in HPLC offers information about the chemical and physical properties of the stationary and the mobile phase, explaining the specific and non-specific interactions occurring in the HPLC column.[8–13] Evaluations of the variations of free Gibbs enthalpy (ΔG^0), the standard enthalpy (ΔH^0), and entropy (ΔS^0) are determined from the chromatographic data, and usually they have to fulfil the following conditions: $\Delta H^0 < 0$ (typically of order of kJ/mol) and entropy $\Delta S^0 < 0$ (typically of order of J/mol K).[14] Their interval of variation differs between different types of retention mechanisms. In order to have $\Delta G^0 < 0$, which is the main thermodynamic condition for the entire process, it is necessary to have $\Delta H^0 < T\Delta S^0$ (the process is driven by enthalpy). In rare situations when ΔH^0 is positive, the condition is that the absolute value of ΔH^0 is smaller than $T\Delta S^0$, and ΔS^0 is also positive. In this last case, the process is apparently driven by entropy. The main controlled parameter that is varied in sequences of chromatographic experiments is column temperature (T), and in some circumstances the composition of the mobile phase.[15]

THEORETICAL ASPECTS

For an assumed unique partition equilibrium of a solute between two phases, the mobile and stationary phase, a partition equilibrium K_d can be assigned to the process that is temperature dependent as follows:

$$K_d = e^{-\frac{\Delta G^0}{RT}} \quad (1)$$

here, ΔG^0 represents the variation in the standard free Gibbs enthalpy for the partition process, T is absolute temperature (in K), and R is the gas constant (8.314 J/mol·K). According to this equation, the retention in HPLC decreases with the increase of the column temperature.

The retention factor k' measured for a solute is related to K_d by the formula:

$$k' = K_d \Phi \quad (2)$$

In eq. 2 the parameter Φ represents the phase ratio, defined as the ratio between the stationary phase volume (V_s) and the volume of mobile phase (V_m) from the HPLC column, i.e. $\Phi = V_s/V_m$. With the aid of this formula, eq. 1 can be written in the form:[16,17]

$$\ln k' = -\frac{\Delta H^0}{R} \cdot \frac{1}{T} + \frac{\Delta S^0}{R} + \ln \Phi \quad (3)$$

This dependence is known as van't Hoff equation and describes the dependence of the retention factor with column temperature T, where ΔH^0 is the variation of standard enthalpy for the transfer of solute from mobile to the stationary phase, and ΔS^0 represents the variation of standard entropy associated to this process.

Equation van't Hoff can be effectively used for the thermodynamic evaluation of the retention process in HPLC by studying experimentally the dependence of ln k' of a solute on $1/T$. Such linear equation can be simplified as:

$$\ln k' = a + b \cdot \frac{1}{T} \quad (4)$$

From the linear plots, the parameters *a* and *b* can be calculated, and with their values, the thermodynamic parameters ΔH^0 and ΔS^0 can be determined by means of formulas:

$$\Delta H^0 = -b \cdot R \quad (5)$$

$$\Delta S^0 = R \cdot (a - \log \Phi) \quad (6)$$

This approach is typically applied in characterizing from a thermodynamic point of view, the various retention mechanisms in HPLC.[18–20] In practice, the linearity between ln *k*' and 1/*T* is not always obeyed.[21–23] Various causes have been suggested that may explain the nonlinear van't Hoff dependencies, such as secondary equilibria involving solutes,[21,22,24] multiple retention mechanisms occurring during the elution process[24,25] or multiple interactions between analyte and stationary phase.[26,27] Another cause could be the variation of phase ratio Φ with temperature,[28–30] but this aspect has been less extensively studied by the literature. The thermodynamic approach by means of van't Hoff analysis is also used for the characterization of new stationary phases,[11,31] or for evaluation of complex mobile phase compositions.[32,33]

For non-linear dependence between ln *k*' and 1/*T*, the relationship between them is described by a polynomial equation written as following:[16]

$$\ln k' = a' + b' \frac{1}{T} + c' \frac{1}{T^2} \quad (7)$$

This time, the thermodynamic parameters ΔH^0 and ΔS^0 become temperature-dependent, being denoted by $\Delta H(T)$ and $\Delta S(T)$, and having the following expressions:[25]

$$\Delta H(T) = -R\left(b' + \frac{2c'}{T}\right) \quad (8)$$

$$\Delta S(T) = R\left(a' - \ln \Phi - \frac{c'}{T^2}\right) \quad (9)$$

All these situations are discussed further, for three complex molecules that are involved in various structural modifications resulted by changing the mobile phase composition.

When the polynomial eq. 7 has a maximum on the 1/*T* interval verified experimentally (illustrated in Fig. 2), this point can be calculated from the condition:

$$\frac{\partial(\ln k')}{\partial\left(\frac{1}{T}\right)} = 0 \quad (10)$$

Applied to eq. 7, the result is the following:

$$\left(\frac{1}{T}\right)_{\ln k' = maximum} = -\frac{b'}{2c'} \quad (11)$$

At the maximum point, the variation of $\Delta H(T)$ and $\Delta S(T)$ becomes:

$$\Delta H = 0 \quad (12)$$

$$\Delta S = R\left(a' - \ln \Phi - \frac{b'}{4c'}\right) \quad (13)$$

From this maximum point on, the retention process is apparently governed by entropy, when the situation can be considered as an *anti*-van't Hoff dependence that fulfils the following condition:

$$0 < \Delta H < T\Delta S \quad and \ \Delta G < 0 \quad (14)$$

Up to this point, the retention process is governed by enthalpy, when the situation is described by van't Hoff rule that fulfils the following condition:

$$0 > \Delta H > T\Delta S \quad and \ \Delta G < 0 \quad (15)$$

Both situations were observed by studying two of the studied compounds and discussed further.

When the polynomial equation 7 has a minimum on the 1/*T* interval verified experimentally, the above discussion is the same, now the significance of the two temperature domains being inversed: the first domain up to the minimum corresponds to *anti*-van't Hoff behavior, while from the minimum point on the dependence ln *k' versus* 1/*T* corresponds to the van't Hoff rule.

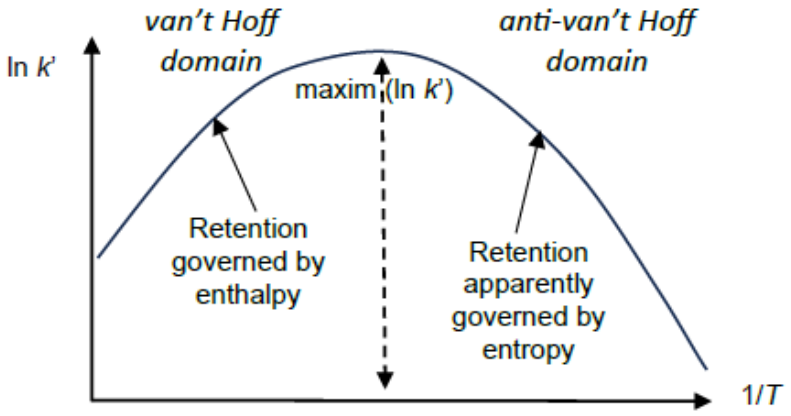

Fig. 2 – Hypothetical polynomial variation of ln *k*' as a function of 1/*T* for a van't Hoff and *anti*-van't Hoff behavior of a separation system.

EXPERIMENTAL

The experimental part was performed on a HPLC system (Agilent 1100 Series) consisting of the following modules: degasser (G1322A), binary pump (G1312A), autosampler (G1329A), column thermostat (G1316A), and variable wavelength detector (G1314A), assisted by Agilent Chemstation software revision B.03.02 for data acquisition. The studies were performed on three silica-based columns with the following characteristics:

– Gemini 5u C18 (produced by Phenomenex): 100 mm length, 4.6 mm internal diameter, 5 μm particle size;

– Ultisil XB-C18 (Welch): 150 mm x 4.6 mm, 5 μm;

– Luna C8 (Phenomenex); 150 mm x 4.6 mm, 3 μm.

All runs were performed in isocratic mode with a flow-rate of 1 mL/min, at various temperatures between 25–50°C. Acetonitrile (ACN) and methanol (MeOH), HPLC (gradient) grade and purchased from Sigma-Aldrich (Germany), were used as individual organic modifiers of the mobile phase composition. Different mobile phase compositions were generated using the pumping capability of the HPLC instrument (40% ACN and 60% aqueous component, v/v; 50% MeOH and 50% aqueous component, v/v). Individual solutions in methanol, containing 550 μg/mL sildenafil, 400 μg/mL vardenafil, and 300 μg/mL tadalafil were injected, their injection volume being 1 μL. Chromatograms were monitored in UV at 254 nm.

The control of pH of the aqueous component was essential for the entire study, and the aqueous buffer solutions were obtained and verified experimentally under the following conditions:

– a solution of 0.1% H_3PO_4 for pH = 2.5;

– a solution of 10 mmoles/L NaH_2PO_4 for pH = 5.3;

– a buffer obtained from 10 mmoles/L NaH_2PO_4 solution adjusted with 10 mmoles/L Na_2HPO_4 solution for pH = 6.3;

– a solution of 10 mmoles/L Na_2HPO_4 for pH = 7.5.

The retention factor k' was calculated with the formula $k' = (t_R - t_0)/t_0$, with dead time t_0 determined from the retention time of uracil as tracer injected under the same conditions as the studied solutes.[34–36]

RESULTS AND DISCUSSION

The first studied solute was sildenafil with mobile phases containing an aqueous component with pH indicated in the Experimental section. The plots representing the dependence between ln k' and $1/T$ for sildenafil at these pH values and using individual ACN or MeOH as organic modifier in mobile phase compositions (indicated in Experimental section), for the three HPLC columns, are grouped as graphs in Fig. 3. This compound is involved in several equilibria that are changing the molecular structure in dependence of pH. These structures for sildenafil species, depending on the pH, are represented in Fig. 4. Based on the theoretical framework developed in a previous paper,[25] these equilibria have an influence on the van't Hoff dependences, producing their deviation from linearity. Moreover, the possibility of existing in different tautomeric structures may also have an influence on the non-linear van't Hoff plots, as suggested for other studied compounds.[21,22] Three tautomeric structures of sildenafil (Fig. 5) have different water/octanol partition constants (log K_{ow}), as one of the main molecular descriptor for the retention in RP-HPLC,[37–39] and different van der Waals (vdW) surfaces,[22] and consequently different interactions with the two phases. The values estimated for log K_{ow} of different tautomeric forms of sildenafil, and their molecular surface areas, are indicated in Table 1. The presence of tautomeric forms and their proportion as a function of pH further complicates the HPLC behavior of this compound.

At pH = 2.5, sildenafil is present in almost a unique structure, which is expected to result in a linear van't Hoff plot. This can be verified in Fig. 3 for both organic modifiers and the three utilized HPLC columns. At higher pH values of the mobile phase, deviations from linearity are observed, but differences are noticed between columns and organic modifiers. This can be explained by differences between the three HPLC columns (degree of residual silanols, traces of metallic ions, which can be involved in the retention mechanism).[40] The nature of the organic modifier may influence the real pH of the mobile phase, which is different in the presence of acetonitrile compared to methanol.[41,42]

Generally, the linear dependences between ln k' and $1/T$ were characterized by high values for the coefficient of determination ($R^2 > 0.9000$). When polynomial regressions applied to the dependence ln k' and $1/T$ gave better values for R^2 than linear regressions, or in some situations when the trend of these graphs was in favor of polynomial dependence, this type of dependence was chosen and used in data processing.

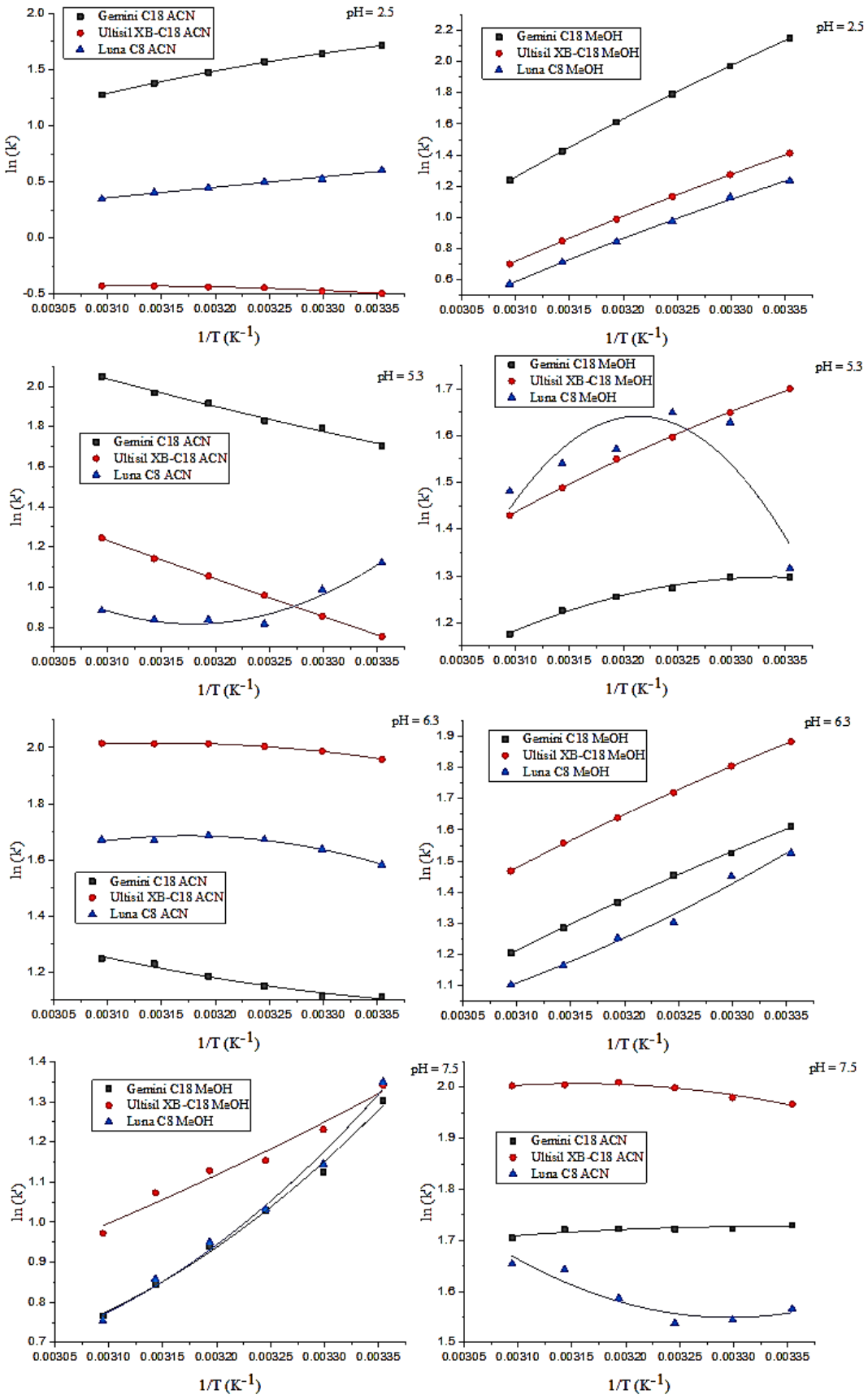

Fig. 3 – Dependences between ln *k'* and 1/*T* for sildenafil for the three columns and two organic modifiers (ACN and MeOH), at indicated pH values of the aqueous component in mobile phase.

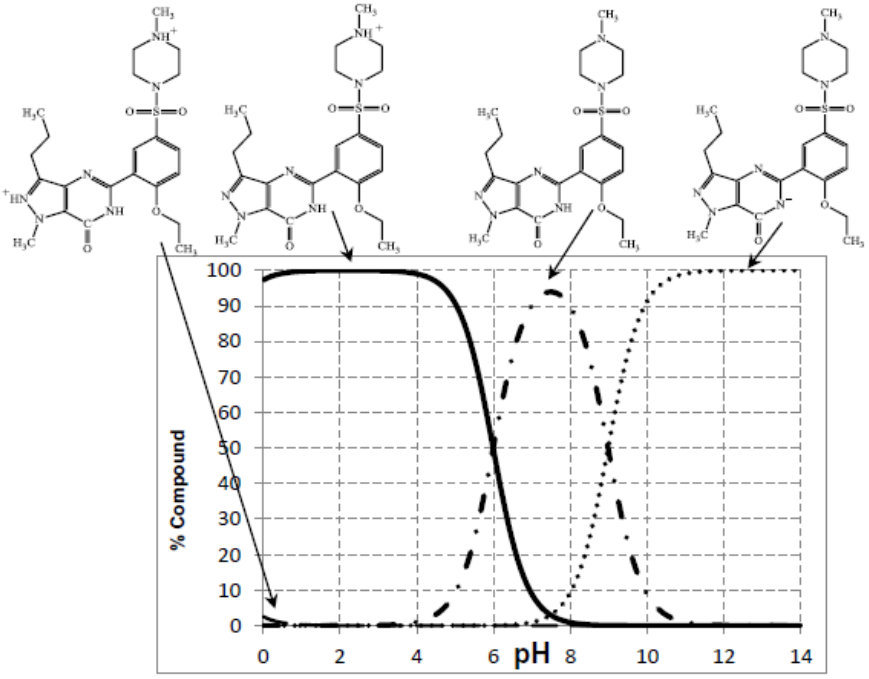

Fig. 4 – Structural modifications of sildenafil in the pH interval of [0, 14] and their proportions with pH variation.

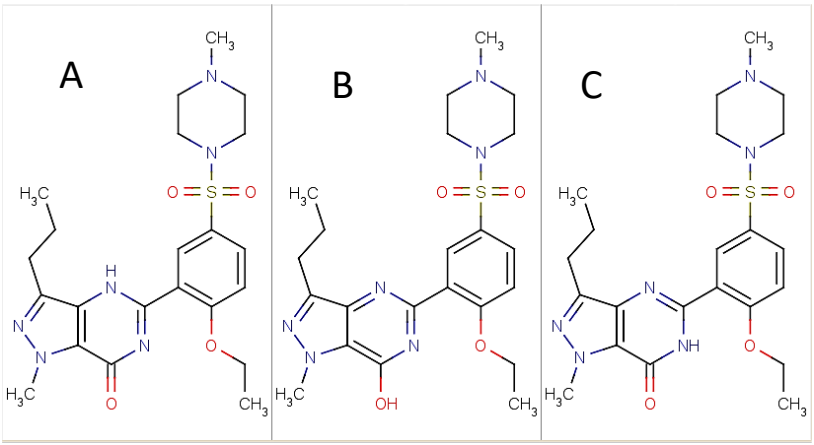

Fig. 5 – Tautomeric forms of sildenafil.

Table 1

Calculated values for log K_{ow} and molecular surface areas for different tautomers of sildenafil as obtained using MarvinSketch 23.14 program (Chemaxon.com).

Tautomer A	log K_{ow} = 1.87	vdW Surface = 705.75 Å^2
Tautomer B	log K_{ow} = 2.96	vdW Surface = 713.09 Å^2
Tautomer C	log K_{ow} = 1.35	vdW Surface = 705.77 Å^2

The secondary equilibria associated to different protonation process of solute X leading to different species X_i are characterized by ΔG^0_i. The global free Gibbs enthalpy ΔG of the entire elution process that include ΔG^0 for the primary equilibrium (partition between mobile phase and stationary phase) and ΔG^0_i for each secondary equilibrium[22,25] becomes a complex function of each contribution to the process that finally results in a dependence between ln k' and $1/T$ given by eq. 7. With the aid of eqs. 12 and 13 the values of ΔH, ΔS and ΔG can be computed at different temperatures of the chromatographic column, considering a value Φ = 0.25.[29,30] Some examples from the multitude of data resulted from this study are presented in Table 2, revealing the two domains of ln k' *versus* $1/T$: a van't Hoff behavior resulted for the pH = 2.5, and an *anti*-van't Hoff behavior for pH = 6.3.

Table 2

Thermodynamic parameters at different temperatures associated to the chromatographic partition of sildenafil (Gemini 5u C18, and ACN as organic modifier) at two pH values of the aqueous component.

T (K)	pH = 2.5			pH = 6.3		
	Δ*H* (kJ/mol)	Δ*S* (J/mol·K)	Δ*G* (kJ/mol)	Δ*H* (kJ/mol)	*ΔS* (J/mol·K)	Δ*G* (kJ/mol)
298.15	–9.47	–5.96	–7.69	2.79	30.10	–6.18
303.15	–11.40	–12.39	–7.64	3.67	33.01	–6.34
308.15	–13.27	–18.51	–7.56	4.52	35.78	–6.51
313.15	–15.08	–24.34	–7.46	5.34	38.42	–6.70
318.15	–16.83	–29.89	–7.32	6.13	40.94	–6.89
323.15	–18.53	–35.19	–7.16	6.90	43.34	–7.11

A remark is advanced here, which refers to the positive values for *ΔS* in case of *anti*-van't Hoff behavior. This is a result of computation of the global *ΔS*, which includes the contributions assigned to all equilibria involved in the elution of the solute molecule. The value of *ΔS* for the solute transfer from mobile phase to the stationary phase in always negative, but the compensations brought by secondary processes may lead to a final positive value of *ΔS,* as it is computed from the experimental data.

Structural similarity of vardenafil with sildenafil can result in similar species formed at various pH values of the solvating aqueous medium (see Fig. 6). In an acidic mobile phase, this compound is present in two forms, while at higher pH value two other species can be formed and involved in the chromatographic partition.

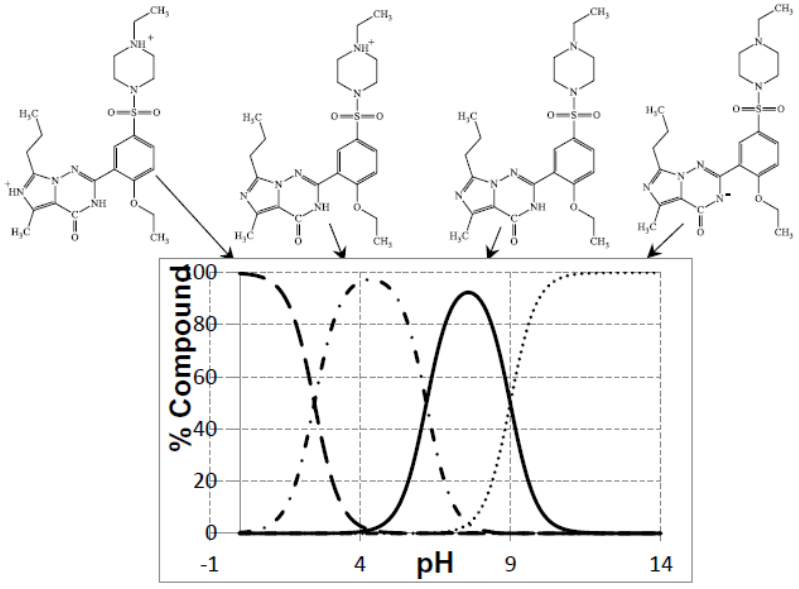

Fig. 6 – Structural modifications of vardenafil in the pH interval of [0, 14] and their proportions with pH variation.

On the other hand, structural similarity of vardenafil with sildenafil is expected to result in similar RP-HPLC behavior, the presence of ethyl group in vardenafil molecule to influence more energetically the retention process compared to methyl group in sildenafil. Indeed, this assumption was confirmed in many experimental conditions, such as the examples presented in Table 3. The conditions used for the results from Table 3 are identical with those presented in Table 2 for sildenafil. Comparing *ΔH*, *ΔS* and *ΔG* in the two cases, one can be observed higher values for vardenafil than for sildenafil, which means a higher interaction of vardenafil with stationary phase than in case of sildenafil. Higher values of *ΔG* for vardenafil is synonymous with higher retention factor *k'*, which was verified experimentally.

Polynomial dependences ln *k'* *versus* 1/*T* were also obtained for vardenafil, as obtained for sildenafil. Unusual plot with minimum point was obtained for vardenafil by using a Luna C8 column and ACN as organic modifier (Fig. 7A), as obtained for sildenafil under the same experimental conditions (see Fig. 3). In the example depicted in

Fig. 7B, the trend is similar for vardenafil with the behavior of sildenafil shown in Fig. 3. Both plots from Fig. 7 indicate the thermodynamic behaviors of this solute under different experimental HPLC conditions, when the retention domains are controlled by enthalpy or by entropy.

Table 3

Thermodynamic parameters at different temperatures associated to the chromatographic partition of vardenafil (Gemini 5u C18, and ACN as organic modifier) at two pH values of the aqueous component

T (K)	pH = 2.5			pH = 6.3		
	Δ*H* (kJ/mol)	Δ*S* (J/mol·K)	Δ*G* (kJ/mol)	Δ*H* (kJ/mol)	Δ*S* (J/mol·K)	Δ*G* (kJ/mol)
298.15	–16.11	–21.47	–9.71	4.25	35.41	–6.31
303.15	–16.69	–23.40	–9.59	4.83	37.34	–6.49
308.15	–17.25	–25.23	–9.47	5.39	39.18	–6.68
313.15	–17.79	–26.98	–9.34	5.94	40.93	–6.88
318.15	–18.32	–28.65	–9.20	6.46	42.60	–7.09
323.15	–18.83	–30.24	–9.06	6.97	44.19	–7.31

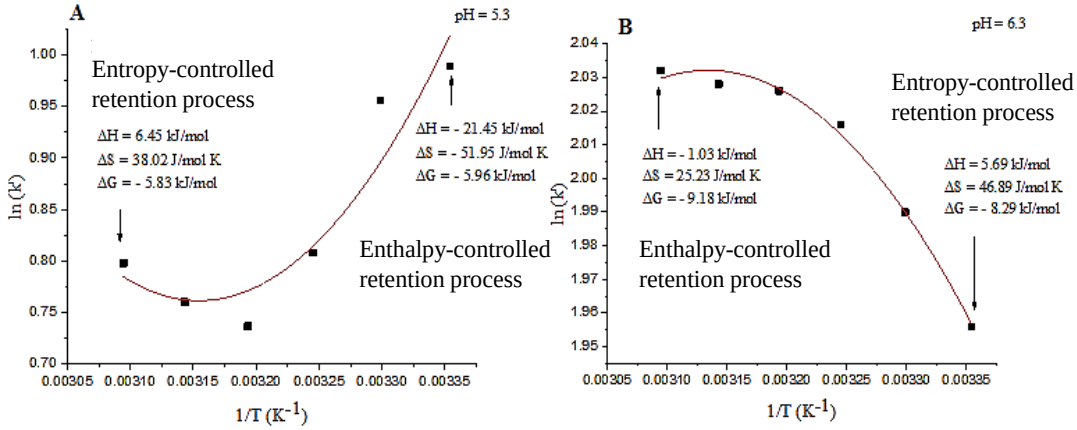

Fig. 7 – Examples of deviation from linear van't Hoff dependence observed for vardenafil by using a Luna C8 column (**A**) and Ultisil XB-C18 (**B**) in case of acetonitrile as organic modifier.

In some cases, for the whole (evaluated) temperature range the dependence of ln *k*' on 1/*T* is dominated by the entropy as indicated in Fig. 8A. In other cases, such as that shown in Fig. 8B the correlation between the two variables, ln *k*' and 1/*T* was poor, but the regression parameters were suitable for computing the thermodynamic parameters *ΔH*, *ΔS* and *ΔG* of the HPLC process. In accordance to the theoretical framework developed in the previous section, the results show the two temperature domains of dependences between ln *k*' and 1/*T*: a van't Hoff domain controlled by enthalpy, and an *anti*-van't Hoff domain controlled apparently by entropy.

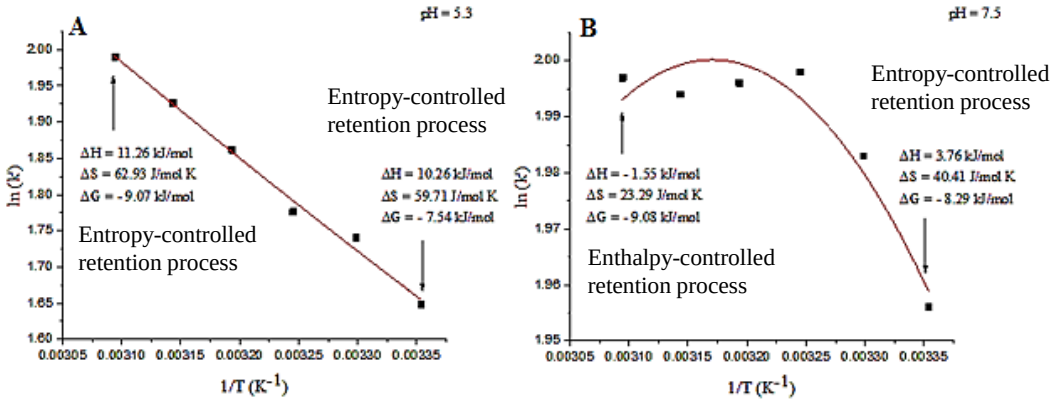

Fig. 8 – Example of various forms of van't Hoff dependence observed for vardenafil by using a Gemini C18 (**A**) and Ultisil XB-C18 (**B**) in the case of methanol as organic modifier.

However, some differences have been noticed for vardenafil when compared with sildenafil. At pH = 7.5, differences between the organic modifiers were obtained for the van't Hoff plots: for ACN vardenafil showed a polynomial ln *k'* *versus* 1/*T* with a maximum on the temperature interval for the three HPLC columns, while for MeOH the dependences were almost linear. This behavior is in contrast to that observed for sildenafil. Differences between their effects have also been reported by the literature. Thus, although vardenafil possesses a chemical structure very similar to sildenafil, experimentally it has been proved that this is much more potent than sildenafil for PDE5 inhibition.[43] Also, theoretical studies have shown that the molecular configuration of vardenafil differs significantly from that of sildenafil when interacting with the structure of PDE5.[5]

Unlike sildenafil and vardenafil, tadalafil has a homogeneous chromatographic behavior for the van't Hoff dependences. This is a consequence of an almost unique molecular structure on the entire pH domain, as can be seen in Fig. 9.

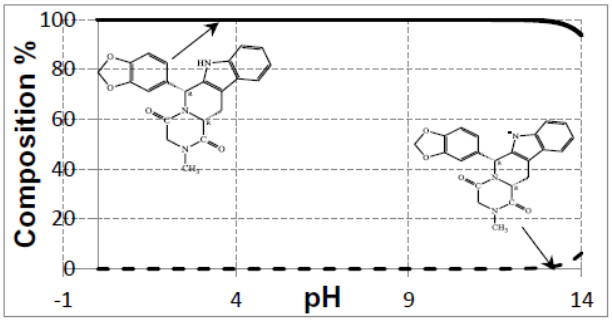

Fig. 9 – Structural modifications of tadalafil in the pH interval of [0, 14] and their proportions with pH variation.

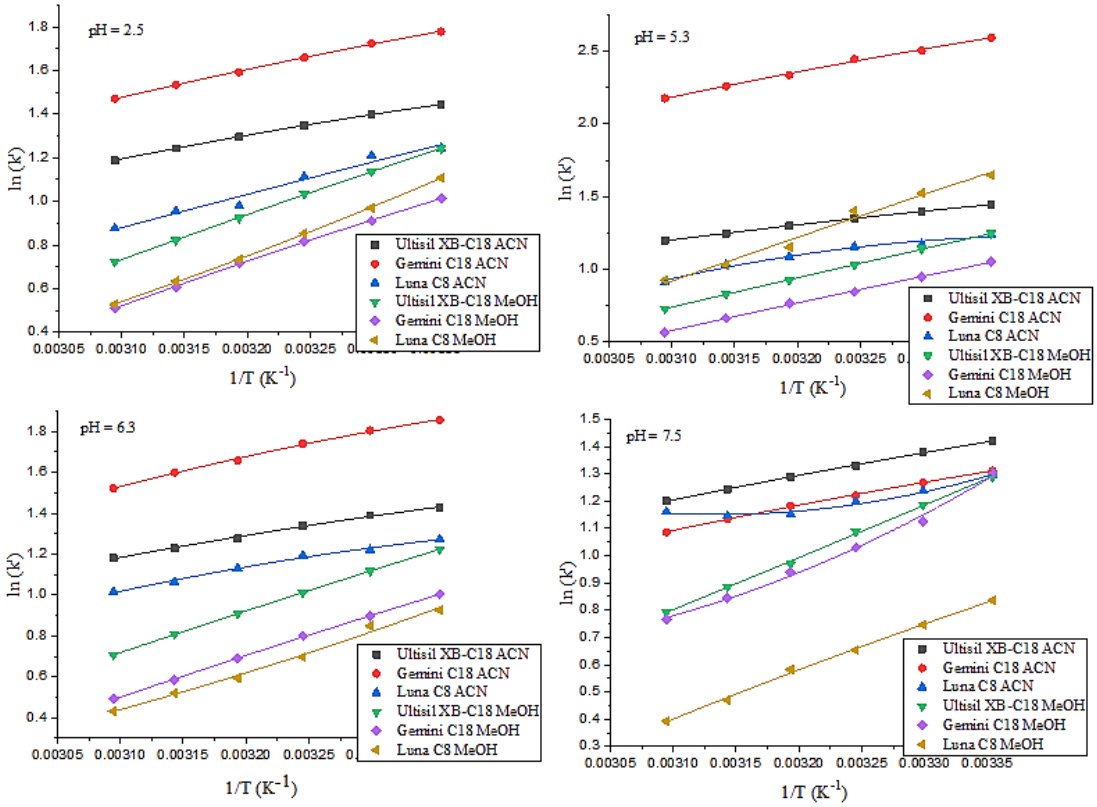

Fig. 10 – Linear dependences between ln *k'* *versus* 1/*T* for tadalafil for all experimental conditions applied in this study.

Regardless of the pH of the mobile phase, the type of organic modifier added to the mobile phase and the three used HPLC column, the dependences between ln *k'* and 1/*T* were linear, with very good correlations as shown in Fig. 10. This homogeneous behavior can be explained by a unique retention mechanism, which

is a consequence of a single structural form of this solute. Only in a single situation, deviation from linearity of ln *k*' *versus* 1/*T* was observed (Luna C8 column and ACN as organic modifier, at pH = 7.5). As a general trend, the retention of tadalafil was higher for ACN than for MeOH, except for the case of using a pH = 7.5 for the aqueous component.

The linear dependence of ln *k*' on 1/*T* for tadalafil is in accordance with the stable structure of this compound in a wide pH range, and the absence of tautomeric forms.

CONCLUSIONS

The present study describes the complex behavior in the HPLC separation process, not following a theoretical ideal one, of two related compounds, sildenafil and vardenafil, and the behavior close to a theoretical expectation of the third compound, tadalafil. The graphical plots ln *k*' *versus* 1/*T* for sildenafil and vardenafil did not show always a theoretical linear dependence, having two distinct domains, one corresponding to van't Hoff dependence and another to *anti*-van't Hoff dependence. Such results were verified by performing repeated experiments to eliminate potential experimental errors. On the other hand, tadalafil, which has a unique structure over a wide pH range, displays the expected behavior. The study may have implications in the prediction of the behavior of these compounds in a physiological environment where the adsorption/desorption processes have similarities with an HPLC separation.

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