ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2012, *57*(2), 131-140

THE INFLUENCE OF MOBILE PHASE pH ON THE RETENTION AND SELECTIVITY OF RELATED BASIC COMPOUNDS IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

Toma GALAON and Victor DAVID*

University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, 90 Sos. Panduri, sect. 5, Bucharest – 050663, Roumania

Received July 20, 2011

Retention change with the pH of the mobile phase is shown for several basic compounds of pharmaceutical importance (drotaverine, ketotifen and chlorphenamine) and some of their related compounds. The partition model is used to explain the pH dependence of the retention for these compounds. Based on the experimental retention data, the dependence between the retention factor (k') and pH of the mobile phase were plotted. Sigmoid shapes were obtained confirming thus the applied theoretical model. The pK_a values were estimated for all the investigated compounds using the inflexion curve parameter. Comparison with theoretical pK_a values obtained with specialized software leads to good correlation for some of the studied compounds.

INTRODUCTION

Retention and selectivity of dissociable compounds in RP-LC is primarily modulated by changing the pH the mobile phase, the nature of the employed buffer or its concentration.[1-5] Thus, the dissociation of the acidic (-COOH, -OH, amide, sulphonamide) or basic (-NH_2, pyridine) moieties can be influenced by the pH of the mobile phase.[6-9] Therefore, this parameter is always taken into consideration for method development of the chromatographic separation of such compounds or in column characterization in RP-LC.[10-12] Organic compounds of pharmaceutical interest are probably the most studied compounds by liquid chromatography, their separation varying from simple mixtures, such as pharmaceutical formulations, to very complex mixtures, such as biological fluids.[13-15] A major interest is also given to the separation of synthesis impurities of the pharmaceutical compounds, or to the compounds resulting from the degradation of pharmaceutical compounds.[16-18] In all these cases, the chromatographic separation is tremendously influenced the pH of the mobile phase, which changes the partition properties of the target compounds between the mobile and stationary phase.[19-21] This process can be described by partition coefficient (denoted by D_{ow}) of studied compounds between aqueous mobile phase and hydrocarbon layer on the surface of stationary phase.[22,23]

The purpose of this paper is to investigate the retention behavior of several pharmaceutical compounds with basic properties by reversed-phase liquid chromatography (RP-LC) and to optimize the separation selectivity by means of the pH parameter. Moreover, the estimation of dissociation constants for these studied compounds was possible by using the dependences between the retention factor (k') and pH value of the mobile phase. The experimental values were further compared with theoretical pK_a values estimated from molecular orbital calculations with the aid of Marvin Beans software version 5.4.0.0 (ChemAxon, Hungary).[24]

EXPERIMENTAL

An Agilent 1100/1200 series LC system consisting of the following modules: degasser (G1322A), binary pump

* Corresponding author: Vict_David@yahoo.com

(G1312A), thermostated autosampler (G1329A/G1330B), column thermostat (G1316A) and diode-array detector (G1315B) were used in the present study.

The pH retention studies were conducted with distinct LC methods for each group of compounds depending on their specific hydrophobicity and basicity.

Drotaverine and its related compounds were chromatographed using an end-capped Zorbax XDB C18 column from Agilent (150 mm length, 4.6 mm internal diameter and 3.5 µm particle size) thermostated at 20°C. The mobile phase consisted of aqueous ammonium formate buffer (25 mM ammonium formate brought to pH with formic acid or ammonia) and acetonitrile in the ratio 60 / 40 (v/v). The investigated pH range was between 3.0 and 6.5. Flow-rate was 1.3 mL/min. The injection volume was 10 µL of standard solution containing 1500 µg/mL drotaverine hydrochloride dissolved in 0.2% aqueous phosphoric acid. UV detection was done at 244 nm.

Ketotifen and its related compounds were studied on an end-capped Purospher STAR RP-18e column from Merck (125 mm length, 4.0 mm internal diameter and 5 µm particle size), thermostated at 20°C. The mobile phase consisted of aqueous 0.1% triethylamine brought to pH with phosphoric acid and acetonitrile in the ratio 60 / 40 (v/v). The investigated pH range was between 7.0 and 8.5. Flow-rate was 1.5 mL/min. The injection volume was 20 µL of a mix standard solution containing 20 µg/mL ketotifen, nor-ketotifen, ketotifen impurity A, B, D and G dissolved in mobile phase. UV detection was done at 297 nm.

Chlorphenamine and its related compounds were chromatographed using an end-capped Chromsep SS Inertsil ODS-2 column from Varian (250 mm length, 4.6 mm internal diameter and 5 µm particle size), thermostated at 50°C. The mobile phase consisted of acetonitrile and aqueous 75 mM $NH_4H_2PO_4$ buffer brought to pH with phosphoric acid, in the ratio 20 / 80 (v/v). The studied pH range was very narrow: between 2.6 and 3.2. Flow-rate was 1.2 mL/min. The injection volume was 20 µL; the injected solution was 4000 µg/mL chlorphenamine maleate in mobile phase, which was previously stressed in highly basic and oxidizing conditions (2 M NaOH and 30% H_2O_2) in order to generate several degradation compounds. UV detection was done at 225 nm.

All solvents were HPLC grade from Merck (Darmstadt, Germany). Reagents were analytical grade from the same producer. Water for chromatography (resistivity minimum 18.2MΩ and 170 TOC maximum 30 ppb) was obtained within the laboratory by means of a TKA Lab HP 6UV/UF instrument. Drotaverine hydrochloride was purchased from Apeloa (China), while chlorphenamine maleate, ketotifen fumarate, ketotifen impurities A, B, D, G and nor-ketotifen were purchased from European Pharmacopoeia, Council of Europe.

RESULTS AND DISCUSSION

Most of the pharmaceutical compounds contain acidic or basic functional groups whose dissociation process can be observed from variation of their retention with change of the pH value of the aqueous component in mobile phase. However, such studies are limited by the narrow pH interval where the stationary phase can be employed (*i.e.* 2 – 8). Therefore, in this domain the basic functional groups can be involved in the dissociation process more than the acidic groups, which require for their dissociation a higher value of the pH of the aqueous component. The studied compounds in this paper are basic compounds as can be seen from structures given in Fig. 1.

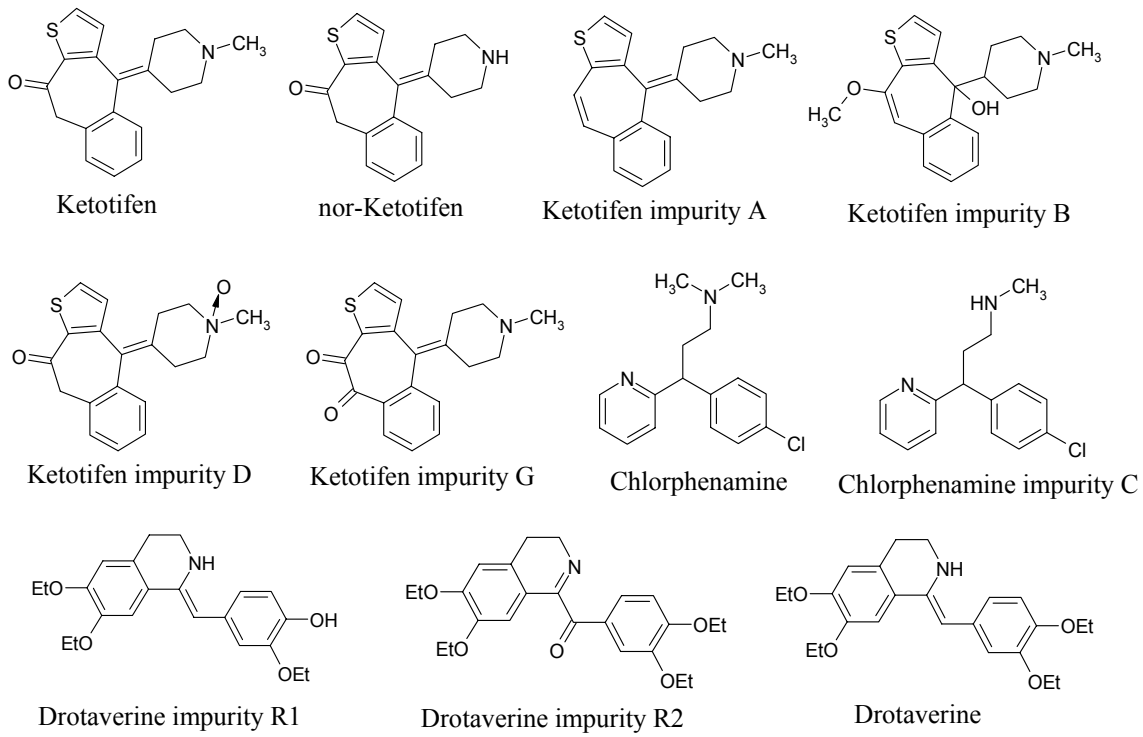

Fig. 1 – Molecular structures of ketotifen, chlorphenamine, drotaverine and their related impurities.

Modeling retention in RP-LC function of the pH parameter requires establishing a functional relationship between the retention factor (k') of a compound and the pH of the mobile phase (considered as given by the aqueous component). Considering the most important models used to explain the retention mechanism in RP-LC, partition and adsorption, it appears that partition accounts better for the retention variation of ionic or dissociable compounds with mobile phase pH variation. The mathematical relationship between retention and pH was initially developed by Horvath *et al*. for different types of solutes (monoprotic or diprotic acids or bases) based on the acid-basic equilibria and solute – stationary phase association equilibria.[1] Combining the expressions of equilibrium constants and dissociation constants Horvath *et al.* obtained the following relationships between retention and pH valid for a monoprotic weak acid and a monoprotic weak base, respectively:

$$k' = \frac{k'_{HA} + k'_{A^-} \times 10^{(pH-pKa)}}{1 + 10^{(pH-pKa)}} \quad (1)$$

$$k' = \frac{k'_{RNH_2} + k'_{RNH_3^+} \times 10^{(pKw-pKb-pH)}}{1 + 10^{(pKw-pKb-pH)}} \quad (2)$$

where $k'_{HA}, k'_{A^-}, k'_{RNH_2}, k'_{RNH_3^+}$ represent for the retention factors of the nondissociated or dissociated forms of the respective acid or base, pK_a and pK_b are the negative sign decimal logarithm of the acidity / basicity constant and pK_w is the ionic product of water.

From a graphical point of view, the dependence of retention factor and the pH of the mobile phase is a sigmoid type exponential curve. Fig. 2 shows the opposite behavior of retention with pH change between an acid and a base.[25] The acid compound (Fig. 2A) has maximum retention when pH approaches 0 because it exists only in undissociated form (leading to strong interaction with the stationary phase) and minimum retention at pH closed to 14 because it exists only in dissociated form (leading to weak interaction with the stationary phase). On the contrary, a basic compound (Fig. 2B) should be totally protonated at pH close to 0 (minimum retention in the chromatographic column) and should be totally deprotonated at pH 14 (highest retention factor). It is to be emphasized that pH = 0 and pH 14 are discussed as theoretical limits due to the fact that typical stationary phase materials used in RP-LC like modified silicagel are not compatible with these pH extremes.

The inflexion point of the two dependences in Fig. 2 corresponds to a pH equal to the pK_a value of an acidic or a basic compound and corresponds on the ordonate axis to the average of retention factors of the dissociated and undissociated forms for both acid and basic compounds.

Drotaverine and related compounds retention - pH study

The retention study for drotaverine and its related compounds was achieved within the pH range of 3.0 – 6.5 and performed in the view of separation and quantification of drotaverine and related compounds in pharmaceutical formulations. Due to the fact that only two impurities of drotaverine were known and none was available among the large number of related compounds present in the analyzed samples, an arbitrary denomination of the unknown compounds was utilized while the known related compounds, namely drotaverine impurity R1 and R2 were identified by relative retention times with respect to drotaverine peak. The injected samples were always a concentrated solution (1500 µg/mL) of drotaverine hydrochloride. At this level at least 7 other related compounds were visible in the chromatograms, which were nominated as drotaverine impurity 1 ÷ 6 in their elution order, and the seventh compound was nominated by impurity X to distinguish it from the others. Typical chromatograms obtained in the retention study are shown in Fig. 3. As can be seen the retention increased from pH 4.5 to pH 6.0 for drotaverine and related compounds. Retention of impurity X increases significantly with 45% from pH 4.5 to 6.0 leading to a change in elution order.

Based on the experimental retention time, the retention factor was calculated and plotted against the pH of the mobile phase for drotaverine and all related compounds (Figs. 4 and 5).

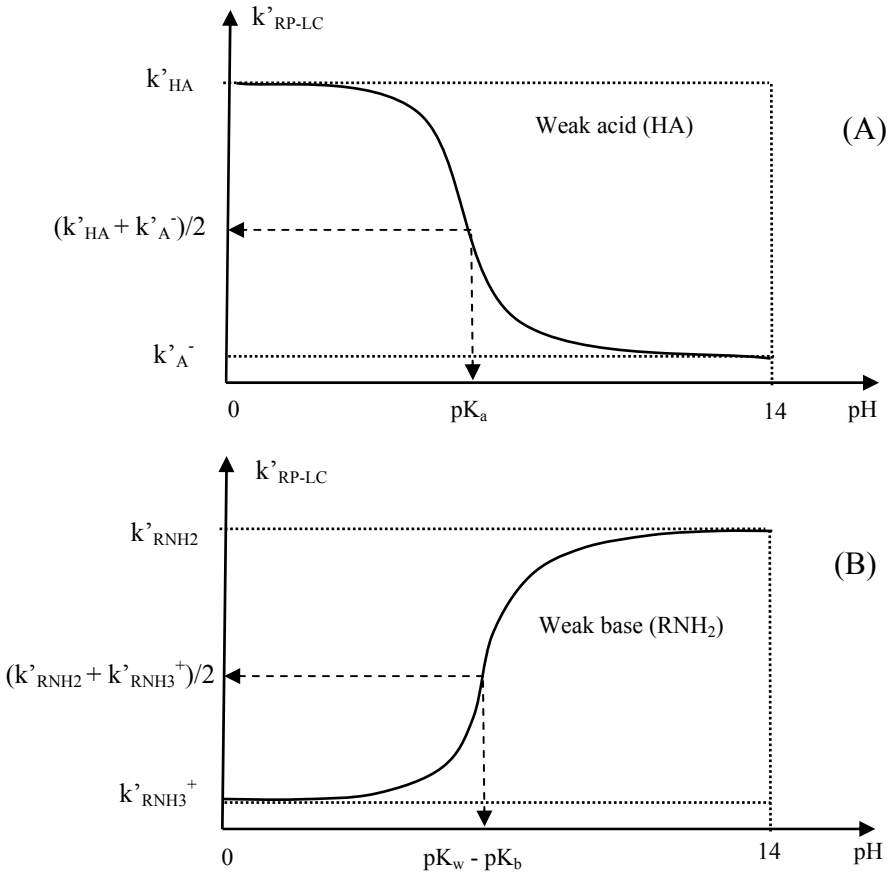

Fig. 2 – Retention dependences expressed as retention factor (k') on the pH of the mobile phase for weak acidic organic compounds (A) and weak basic organic compounds (B).

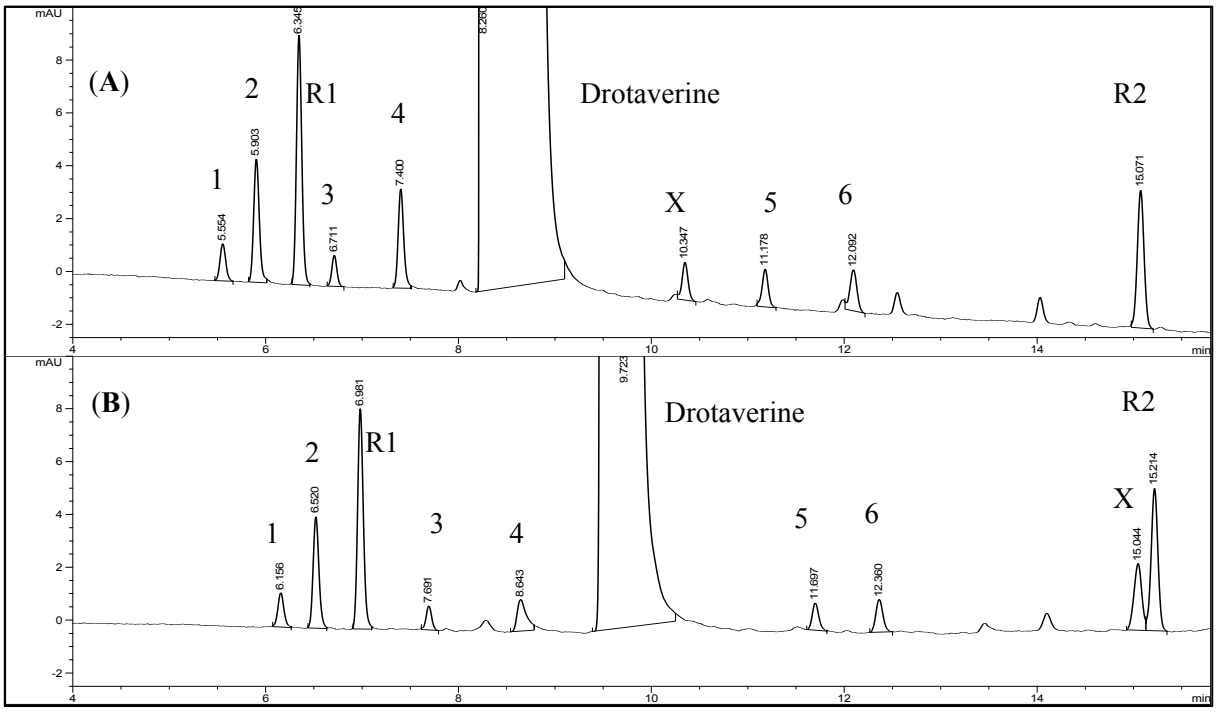

Fig. 3 – Two chromatograms showing variation of retention with pH of the mobile phase for drotaverine and related compounds at (A) pH 4.5 and (B) pH 6.0.

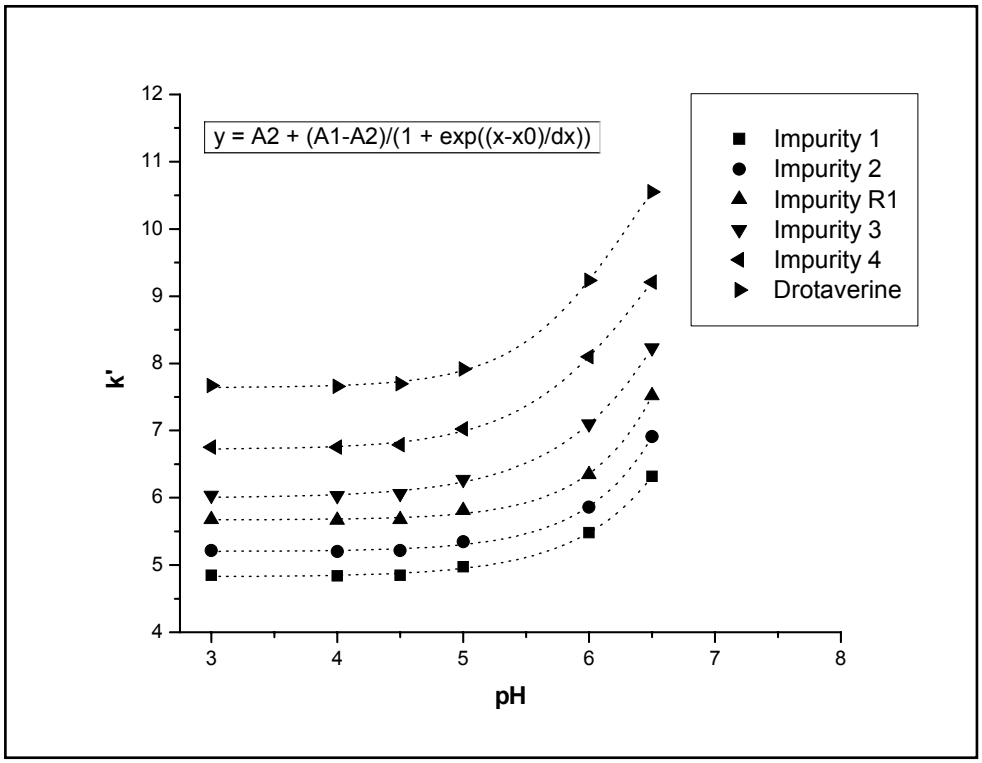

Fig. 4 – Dependence type k' – pH plots for drotaverine and impurities 1, 2, 3, 4 and R1.

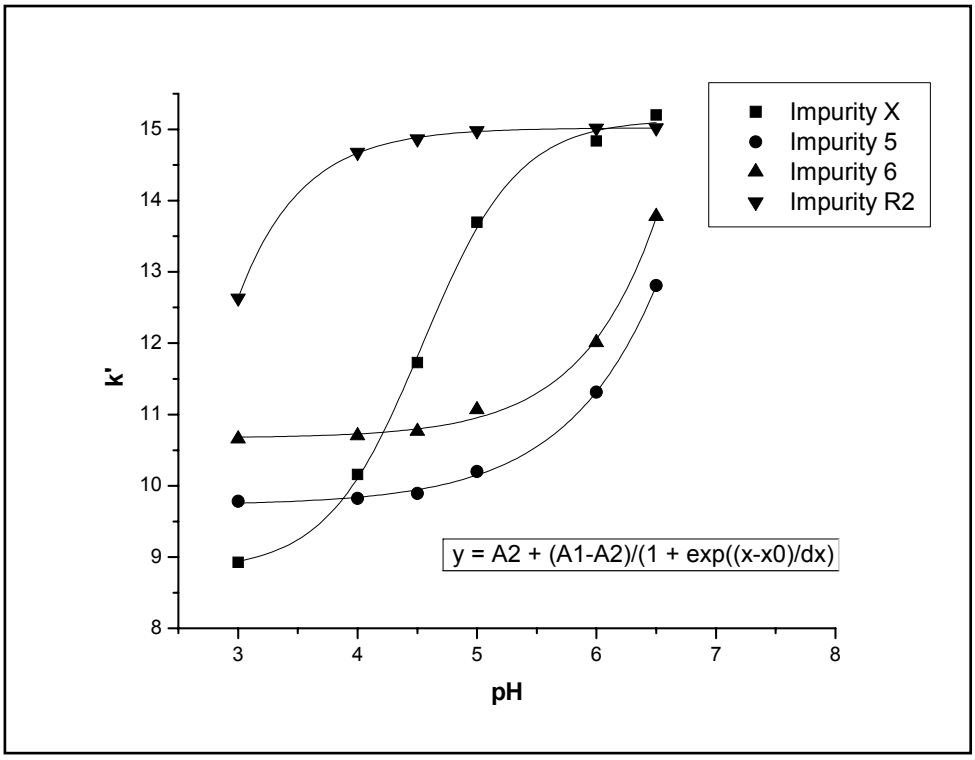

Fig. 5 – Dependence type k' – pH plots for drotaverine impurities X, 5, 6 and R2.

k' – pH plots in Figs. 4 and 5 show a base character for drotaverine and for all its related compounds because of the increase in retention with the increase in mobile phase pH. This behavior gives an indication that all these compounds have basic character.[26] This was confirmed for drotaverine and for impurity R1 by their molecular structures (Fig. 1) containing

secondary amino moieties, which give the basic character for these molecules. The k' – pH plots corresponding to drotaverine, impurity 1 ÷ 6 and impurity R1 show a slight increase in retention from pH 3 to pH 5 and a more important retention increase in the range pH 5 to pH 6.5, as the curve draws nearer towards its inflexion point. Drotaverine impurity R2 has a rather different k'-pH profile, because the pH range 3.0 – 6.5 represents in fact the upper part of the sigmoid curve typical for basic compounds (Fig. 2B) unlike for the previous discussed impurities. The middle region of the sigmoid curve including the inflexion point was obtained only in the case of impurity X as can also be seen in Fig. 5. This figure also shows how the elution order between impurities X and the group of impurities 5, 6 and R2 is changed with increasing pH of the mobile phase. More precisely, impurity X elutes after impurity R2 at pH 6.5, whereas it eluted before the series impurity 5, impurity 6 and impurity R2 at pH 3. In the case of impurity 1 ÷ 4, R1 and drotaverine there are no elution order changes with pH modification in the studied range (Fig. 4), while resolution between these peak pairs is not significantly changed.

The k' – pH plots obtained for drotaverine and related compounds were fitted using the Fit sigmoidal function in Origin 8 program (OriginLab Corporation, USA) which is a Boltzmann exponential function of the type: y = A2 + (A1 - A2)/(1 + exp((x-x0)/dx)). The r-square correlation coefficients and the values for the inflexion points (x0) of the curves are given in Table 1. As previously stated, the inflexion point of the sigmoid curve describing k' – pH plot is an estimation of pK_a value of the respective compound.

The correlation coefficients (r^2) for all drotaverine related compounds were higher than 0.99 which indicates a good data fitting. pK_a values calculated from the inflexion point of k' – pH plots were compared to pK_a values calculated with Marvin Beans software version 5.4.0.0, but only for the three known structures of drotaverine and impurities R1 and R2 (Table 1). For drotaverine the two values are in a good agreement, but for the two impurities there are big differences between theoretical and experimental values. The pK_a values obtained from k' – pH plots for drotaverine and all other impurities except impurity X, should be carefully considered because the pH range was not large enough to include the entire sigmoid curve for these compounds and significant errors in sigmoid curve fitting parameters are possible. For impurity X, the middle region of the curve was included in the studied range but its structure is unknown. Generally, pK_a calculation using RP-LC gives larger errors than classical methods like potentiometry or spectrophotometry.[27,28] One of the most important disadvantages of the LC method is that the pH of the mobile phase is limited by the stability of the stationary phase and thus the pK_a range of values is also limited. Also, the presence of organic modifier in the mobile phase leads to deviations from pK_a values obtained in pure water.[29]

Ketotifen and related compounds retention - pH study

Ketotifen and most of its related compounds have basic character due to secondary or tertiary amino group in their molecule.[30] That was the reason for choosing pH 8 for the LC method dedicated to separation and quantification of ketotifen related compounds in tablet pharmaceutical formulations. When assessing robustness of the method, a small retention – pH study was also achieved in the range pH 7.0 – 8.5. The LC method separated ketotifen and five related impurities (ketotifen impurities A, B, D, G and nor-ketotifen). Two chromatograms obtained in the retention study are shown in Fig. 6.

Table 1

Comparison between theoretical pK_a obtained with Marvin software and experimental pK_a obtained from inflexion of the Boltzmann sigmoid fit for k' - pH plots for drotaverine and impurities

Compound	r^2 (sigmoid fit)	x0 = pK_a (RP-LC)	pK_a (Marvin)
Drotaverine	0.99912	6.35	6.23
Drotaverine impurity R1	0.99691	9.66	7.41
Drotaverine impurity R2	0.99965	0.47	3.36
Drotaverine impurity 1	0.99694	9.08	-
Drotaverine impurity 2	0.99703	9.77	-
Drotaverine impurity 3	0.99740	7.51	-
Drotaverine impurity 4	0.99827	6.52	-
Drotaverine impurity X	0.99629	4.54	-
Drotaverine impurity 5	0.99781	9.60	-
Drotaverine impurity 6	0.99375	10.89	-

Based on the experimental data, k' – pH plots were represented in the studied pH range for ketotifen and all related compounds (Fig. 7). It can be seen from these dependences that retention of basic compounds like ketotifen, impurity A, impurity B, impurity G and nor-ketotifen increases with the increase of mobile phase pH. Retention of impurity D decreases slowly with pH increase, behaving like an acidic compound due to keto and thiazole groups. The most important retention variation with pH in the range 7 – 8.5 can be seen for impurity B and for nor-ketotifen.

The investigated pH range (7.0 – 8.5) is situated close to the pK_a value for these compounds. Below this pH value ketotifen and its related compounds have a low retention and therefore the k' – pH plots for these compounds could not give a good fit by the sigmoid exponential regression. However, by fitting these experimental data with Origin 8 the estimation of the inflexion was estimated and compared to the theoretical pK_a values obtained using Marvin Beans software vers. 5.4.0.0. (Table 2).

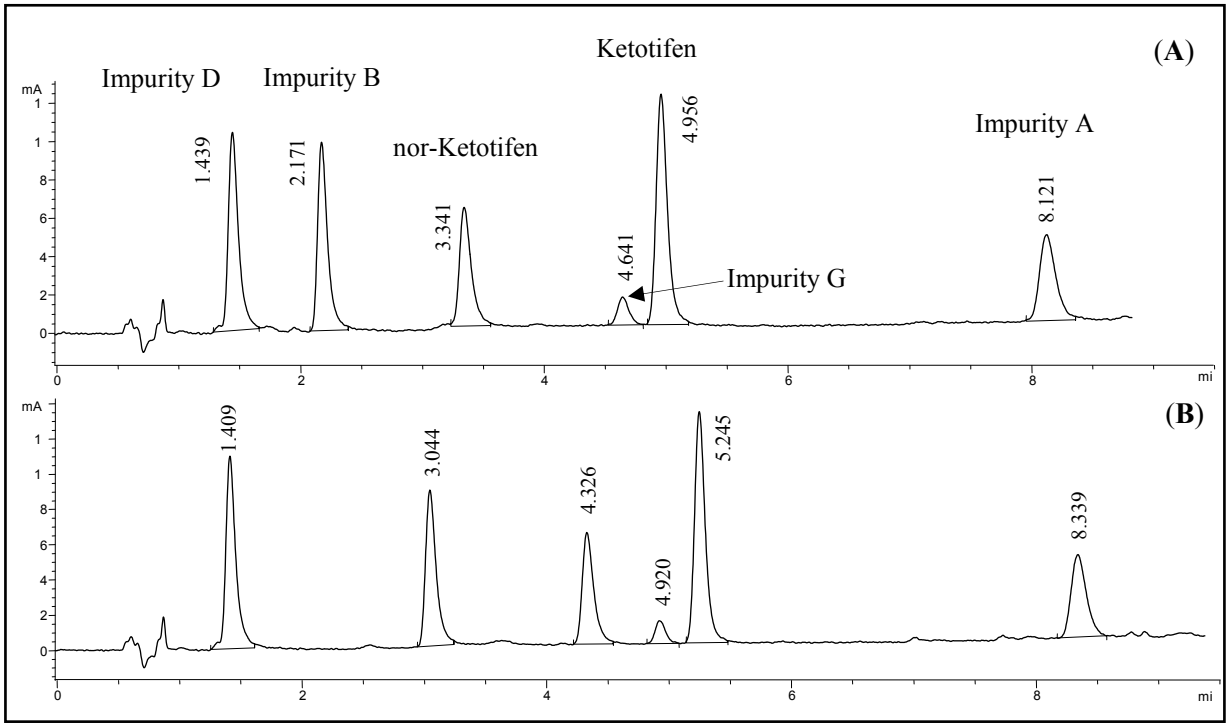

Fig. 6 – Chromatograms showing variation of retention with pH of the mobile phase for ketotifen and related compounds for pH = 7.5 (A) and pH = 8.5 (B).

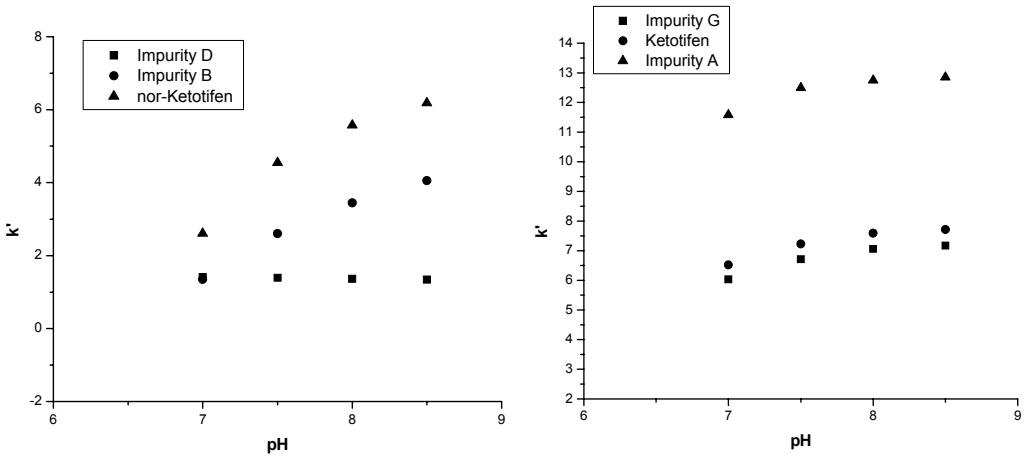

Fig. 7 – Retention factor (k') – pH dependences for ketotifen and its related impurities.

Table 2

Comparison between theoretical pK_a obtained with Marvin software and experimental pK_a obtained from inflexion of the Boltzmann sigmoid fit for k' - pH plots for ketotifen and impurities

Compound	x0 = pK_a (RP-LC)	pK_a (Marvin)
Ketotifen	7.41	7.15
nor-Ketotifen	7.46	9.63
Ketotifen impurity A	7.35	7.91
Ketotifen impurity B	7.56	8.42
Ketotifen impurity G	7.42	6.32

Concerning selectivity aspects with pH in the range 7.0 - 8.5, it was observed from primary retention data that resolution between the peaks corresponding to impurity D and impurity B decreases significantly with pH decrease until the point of coelution of the two compounds at pH 7.0. This observation is confirmed by the k' – pH plot (Fig. 7). Conversely, for the pair nor-ketotifen – impurity G the resolution decreases as the pH increases in the studied range, until a possible coelution at pH values beyond 8.5. All these selectivity changes are caused by the fact that impurity B and nor-ketotifen show a much more important retention variation with pH than impurities A, G and ketotifen do in the studied pH range.

Chlorphenamine and related compounds retention - pH study

Retention - pH study of chlorphenamine and related compounds was performed in the view of assessing the robustness of the LC method for the separation and quantification of chlorphenamine related compounds generated either in the active pharmaceutical ingredient synthesis process or by degradation. Because of the unavailability of chlorphenamine impurities reference standards, a concentrated solution of chlorphenamine maleate (4000 µg/mL) previously stressed in strong oxidative (with 30% H_2O_2) and alkaline conditions (2 M NaOH) was used as system suitability solution to test method robustness and selectivity.[31] The applied stress generated three peaks: chlorphenamine impurity C obtained by N-demethylation of chlorphenamine (the impurity was identified by means of the relative retention time with respect to chlorphenamine) and two unknown impurities, which will be further arbitrarily called impurity 1 and impurity 2.

A narrow pH range was chosen for the robustness study of the method (pH between 2.2 and 3.2) because of the significant retention variation of chlorphenamine, impurity C and impurity 2 (approximately 180% an average retention increase for 0.6 pH units increase). Two overlaid chromatograms are shown in Fig. 8. Retention of impurity 1 was not influenced at all by pH variation, which can be explained by the lack of dissociable groups or that if they exist, they do not dissociate at all in the studied pH range. Based on experimental retention data, the k' – pH plots were represented in Fig. 9. A significant increase in retention of chlorphenamine, impurity C and impurity 2 can be observed from these plots when pH of mobile phase increases from 2.6 to 3.2. In the case of the first two compounds this behavior confirms the basicity of the molecules due to their amino moiety and pyridine ring. The basic groups of the two molecules become increasingly deprotonated as the pH increases, which leads to a higher hydrophobicity and hence a higher chromatographic retention. The same behavior was observed for the unknown impurity 2, and hence the conclusion that this molecule may have similar basic moieties as chlorphenamine or impurity C.

Retention increases significantly as mobile phase pH increases with 0.2 units for chlorphenamine, impurity C and impurity 2, due to their basic character. Retention of impurity 1 is not influenced by pH generating an elution order change between impurity C and impurity 1 from pH 2.6 to pH 2.8.

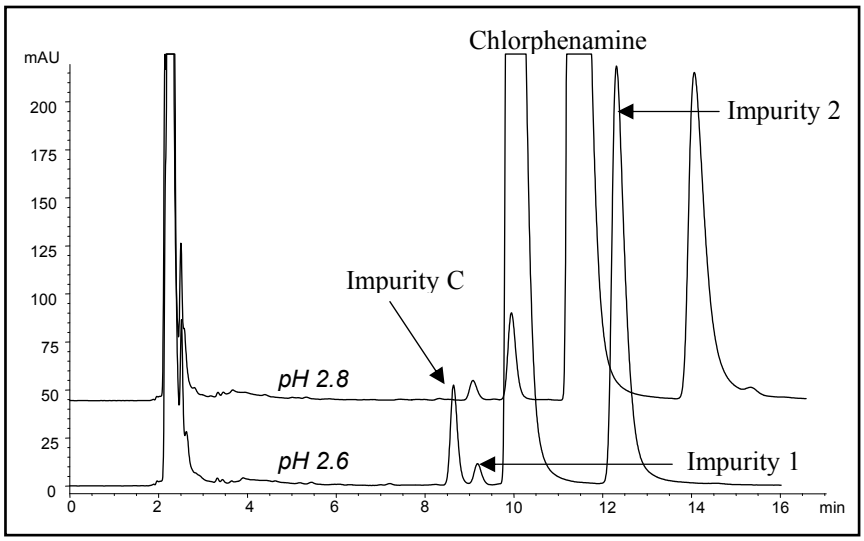

Fig. 8 – Overlaid chromatograms showing variation of retention with pH of the mobile phase for chlorphenamine and related compounds.

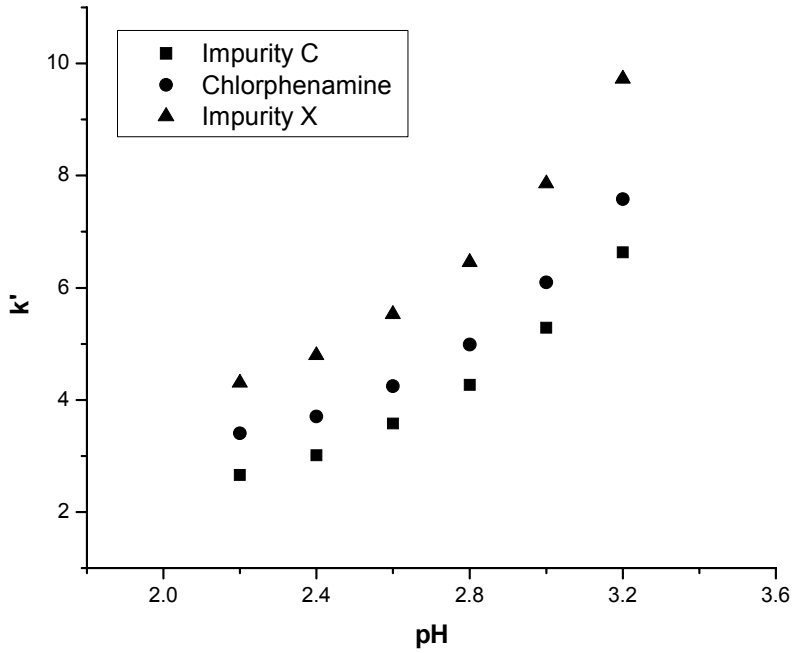

Fig. 9 – Retention factor (k') – pH dependence for chlorphenamine, impurity C and impurity 2.

No sigmoid fitting was possible for the k' – pH plots due to the reduced pH interval available for these compounds. Nevertheless, the large retention variation for the chlorphenamine and impurity C implies that one of the two pK_a values corresponding to the earlier mentioned diprotic bases should be very close to the selected pH range. This was verified by calculating the pK_a values of the two compounds using the Marvin program (Table 3). The pK_a for the pyridine ring in the two molecules is about 2.9 and 3.6, which is nearby the selected pH range. Therefore, by working in RP-LC nearby the pK_a value of a compound this generates a high retention variability for small pH modifications, which leads to a lack of method robustness. The selectivity of the method was also affected by small variations in pH value as can be observed in Fig. 8, namely the elution order between impurity C and the unknown impurity 1 is changed due to the different pH - retention behavior of the two species; retention of impurity 1 remained constant, whereas retention of impurity C increased when pH increased from 2.6 to 2.8.

Table 3

pKa values of chlorphenamine and impurity C obtained with Marvin program

Compound	pKa_1 (pyridine)	pKa_2 (amino)
Chlorphenamine impurity C	2.89	10.17
Chlorphenamine	3.57	9.47

CONCLUSIONS

Experimental modeling of the retention by means of pH of mobile phase allows the prediction of retention for any other pH value inside or outside the studied pH range. This may be useful in developing of an LC method because one could predict the possible elution order changes and selectivity changes during the retention process for the set-up values of the mobile phase parameters. This is expected for ionic or dissociable organic compounds. pK_a values of compounds at different organic content in mobile phase can also be estimated by RP-LC, with the conditions of obtaining many experimental data for their sigmoidal regression. However, in practice this condition cannot be fulfilled when the target compounds have a high hydrophobic character. The value of pH is a powerful parameter for modifying retention, selectivity, elution order of compound in RP-LC. Another important aspect that is to be taken into account when developing a RP-LC method is the fact that working at pH values closed to pK_a of the respective compound, generates large retention time variation with small variations in the mobile pahse pH, meaning lack of robustness or selectivity changes.

Acknowledgements: This work was supported by the strategic grant POSDRU/89/1.5/S/58852, Project "Postdoctoral programme for training scientific researchers" cofinanced by the European Social Found within the Sectoral Operational Program Human Resources Development 2007-2013.

REFERENCES

1. Cs. Horvath, W. Melander and I. Molnar, *Anal. Chem*., **1977**, *49*, 142-154.
2. U.D. Neue, C.H. Phoebe, K. Tran, Y.F. Cheng and Z. Lu, *J. Chromatogr. A*, **2001**, *925*, 49.
3. S. Espinosa, E. Bosch and M. Roses, *J. Chromatogr. A*, **2002**, *947*, 47.
4. P. Wiczling, M.J. Markuszewski and R. Kaliszan, *Anal. Chem*., **2004**, *76*, 3069.
5. Y.V. Kazakevich, *J. Chromatogr. A*, **2006**, *1126*, 232.
6. S. Espinosa, E. Bosch and M. Roses, *J. Chromatogr. A*, **2002**, *964*, 55.
7. S. Espinoza, E. Bosch and M. Roses, *Anal. Chem*., **2000**, *72*, 5193.
8. S. Espinoza, E. Bosch and M. Roses, *Anal. Chim. Acta*, **2002**, *454*, 157.
9. V. David, F. Albu and A. Medvedovici, *J. Liq. Chromatogr. Rel. Technol*., **2004**, *27*, 965.
10. R.J.M. Vervoort, E. Ruyter, A.J.J. Debets, H.A. Claessens, C.A. Cramers and G.J. de Jong, *J. Chromatogr. A*, **2001**, *931*, 67.
11. N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder and P.W. Carr, *J. Chromatogr. A*, **2002**, *961*, 195.
12. J. Layne, *J. Chromatogr. A*, **2002**, *957*, 149.
13. W. Li, J. Zhang and F.L.S. Tse, *Biomed. Chromatogr*., **2011**, *25*, 258.
14. S.D. Brown and T.C. Melton, *Biomed. Chromatogr*., **2011**, *25*, 300.
15. T. Galaon, S. Udrescu, I. Sora, V. David and A. Medvedovici, *Biomed. Chromatogr*., **2007**, *21*, 40.
16. Y. Wu, *Biomed. Chromatogr*., **2000**, *14*, 384.
17. V.K. Vyas, M. Ghate and R.D. Ukawala, *Curr. Pharm. Anal*., **2010**, *6*, 299.
18. P.D. Tzanavaras, *Curr. Org. Chem*., **2010**, *14*, 2348.
19. V. David and A. Medvedovici, *J. Liq. Chromatogr. Rel. Technol*., **2007**, *30*, 761.
20. B. Dejaegher and Y.V. Heyden, *J. Chromatogr. A*, **2007**, *1158*, 138.
21. K.J. Fountain, J. Xu, D.M. Diehl and D. Morrison, *J. Sep. Sci*., **2010**, *33*, 740.
22. L.C. Sander, K.A. Lippa and S.A. Wise, *Anal. Bioanal. Chem*., **2005**, *383*, 646.
23. A. Vailaya and Cs. Horvath, *J. Chromatogr. A*, **1998**, *829*, 1.
24. http://www.chemaxon.com/marvin/help/calculations/partitioning.html
25. V. David and A. Medvedovici, *Rev. Roum. Chim*., **2005**, *50*, 837.
26. T. Galaon and V. David, *J. Sep. Sci*., **2011**, *34*, 1423.
27. J.L. Beltran, N. Sanli, G. Fonrodona, D. Barron, G. Ozkanb and J. Barbosa, *Anal. Chim. Acta*, **2003**, *484*, 253.
28. F.Z. Erdemgil, S. Sanli, N. Sanli, G. Özkan, J. Barbosa, J. Guiteras and J.L. Beltran, *Talanta*, **2007**, *72*, 489.
29. M. Roses, F. Rived and E. Bosch, *J. Chromatogr. A*, **2000**, *867*, 45.
30. M. Elsayed, *Drug Develop. Ind. Pharm.,* **2006**, *32*, 457.
31. I.M. Palabiyik and F. Onur, *Chromatographia*, **2007**, *66*, S93.