Dedicated to the memory of our former teacher, Professor Grigore Popa

PARAMETERS INFLUENCING THE RETENTION MECHANISM OF RALOXIFEN AND ITS RELATED IMPURITIES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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abstract: The main parameters influencing the retention of Raloxifen and its related impurities in reversed-phase liquid chromatography using a monolithic column were studied. Among them the organic modifier content, *p*H, and temperature were studied for developing an optimum high-performance liquid chromatography with diode-array detection method in pharmaceutical samples.

keywords: Raloxifen; retention mechanism; hydrophobicity; liquid chromatography

Introduction

Optimization of the separation in reversed-phase liquid chromatography (RP-LC) of ionizable compounds is usually focused on the following parameters of the mobile phase: organic modifier, *p*H and ionic strength of the aqueous component. On the other hand, the optimization of the separation will take into account the parameters belonging to the analytes of interest and characterizing the stationary phase. Combining these parameters into a single model in order to predict the LC separation is rather difficult, and up to the present several attempts have been advanced [1-4].

In this paper the main parameters that influence the retention of a new drug (Raloxifen) and its related impurities were studied in order to develop an optimized method for their HPLC-DAD determination in pharmaceutical mixtures. Raloxifen hydrochloride is relatively a new drug, which has not been included up to now in International Pharmacopoeias (USP, BP or EP). Its structure as well as the structures of four main related impurities resulted from synthesis procedure are given in Fig. 1.

Experimental

Experiments were performed with an Agilent 1100 liquid chromatograph built-up from a quaternary pump, autosampler, column thermostat, degasser and diode-array detector

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(DAD). Chromatographic data were acquired by means of the Chemstation software (Agilent Technologies). A Chromolith Performance RP-18e column (100 mm length; 4.6 mm i.d.), purchased from Merck (Darmstadt – Germany) was used. The column temperature was set up at 25°C. UV detection was achieved at 240 and 284 ± 2 nm (reference wavelength: 480 ± 10 nm). Flow-rate of the mobile phase was 2.0 mL/min.

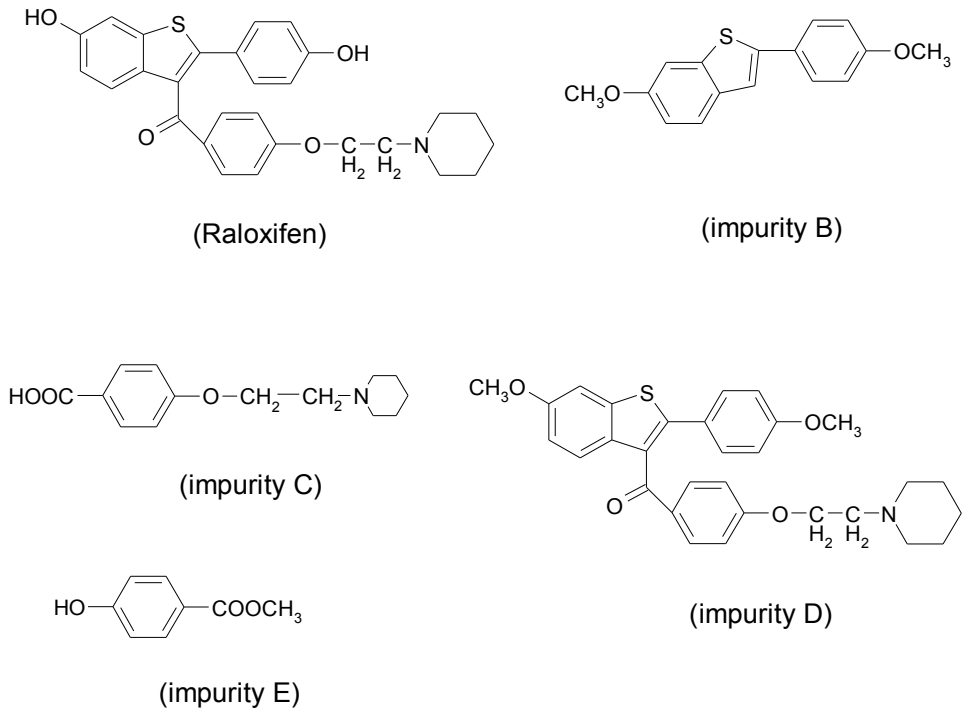

Fig. 1: *Structure of Raloxifen and its four related impurities.*

Table 1. Elution gradient program.

Time (min)	% Aqueous component (solvent A)	% Methanol (solvent B)
0	75	25
2,5	60	40
3,5	35	65
20	15	85

The mobile phase consisted of methanol as organic modifier and the aqueous component: buffer solution containing 0.1% H_3PO_4 adjusted to *p*H = 7.5 with triethylamine (TEA). A gradient elution was applied according to the Table 1. Injection was done automatically for a volume of 10 µL. A methanol solution containing 4 µg mL^{-1} of each analytes of interest was used in this study.

Application

*p*H dependence

The reversed-phase (RP) mechanism of an ionisable analyte in LC is strongly influenced by the *p*H-value of the aqueous component of the mobile phase. The retention of acid analytes is enhanced by decreasing the *p*H of the aqueous component to the acidic domain, while the retention of basic analytes is increased by increasing the *p*H value, but not more than 9 (the analytical column is damaged). The primary equilibrium of an acid compound R-OH that takes place in the mobile phase is following:

$$\text{R-OH} \leftrightarrow \text{R-O}^- + \text{H}^+ \quad K_a = \frac{[\text{RO}^-][\text{H}^+]}{[\text{ROH}]} \tag{1}$$

One results that:

$$\frac{[\text{RO}^-]}{[\text{ROH}]} = \frac{K_a}{[\text{H}^+]} \tag{2}$$

According to the major relationship in LC:

$$k' = \alpha \cdot k'_{\text{ROH}} + (1-\alpha) \cdot k'_{\text{RO}^-} \tag{3}$$

where *k*' is the capacity factor, defined as $(t_r - t_d)/t_d$, t_r – the retention time and t_d – the dead time of the separation; the indexes refer to the species involved in the LC separation. α represents the fraction of the analyte R-OH as following:

$$\alpha = \frac{[\text{ROH}]}{[\text{ROH}]+[\text{RO}^-]} = \frac{[\text{H}^+]}{[\text{H}^+]+K_a} \tag{4}$$

and consequently (1 – α) is the fraction of R-O^-.

Combining the above relationships, one obtains that the capacity factor of an analyte ROH in reversed-phase mechanism is given by the next equation:

$$k' = \frac{1}{1+10^{p\text{H}-p\text{K}_a}}\left(k'_{\text{ROH}} + k'_{\text{RO}^-} \cdot 10^{p\text{H}-p\text{K}_a}\right) \tag{5}$$

According to this equation the contribution of RO^- to the retention of the acidic species is lower at low *p*H values (acid mobile phase) and $p\text{H} < p\text{K}_a$. For higher *p*H-values the contribution of this species becomes significant and thus the acid compound has a low retention. From the studied compounds only Raloxifen, impurity C and impurity E are influenced by the *p*H-value of the aqueous component. However, Raloxifen and impurity C contain a secondary amine also, and thus the influence of *p*H on the functional groups will be opposite. Therefore, only the impurity E can be observed as having retention depending on *p*H, as all acid-like solutes behave in reversed-phase LC. The overall effect of *p*H on their retention can be deduced from Fig. 2, while the dependence of the capacity factor on *p*H for impurity E is represented in Fig. 3. According to these data one results that low variation of *p*H-value of the aqueous component in the mobile phase (due to possible errors in obtaining the buffer solution) do not influence significantly the main chromatographic parameters.

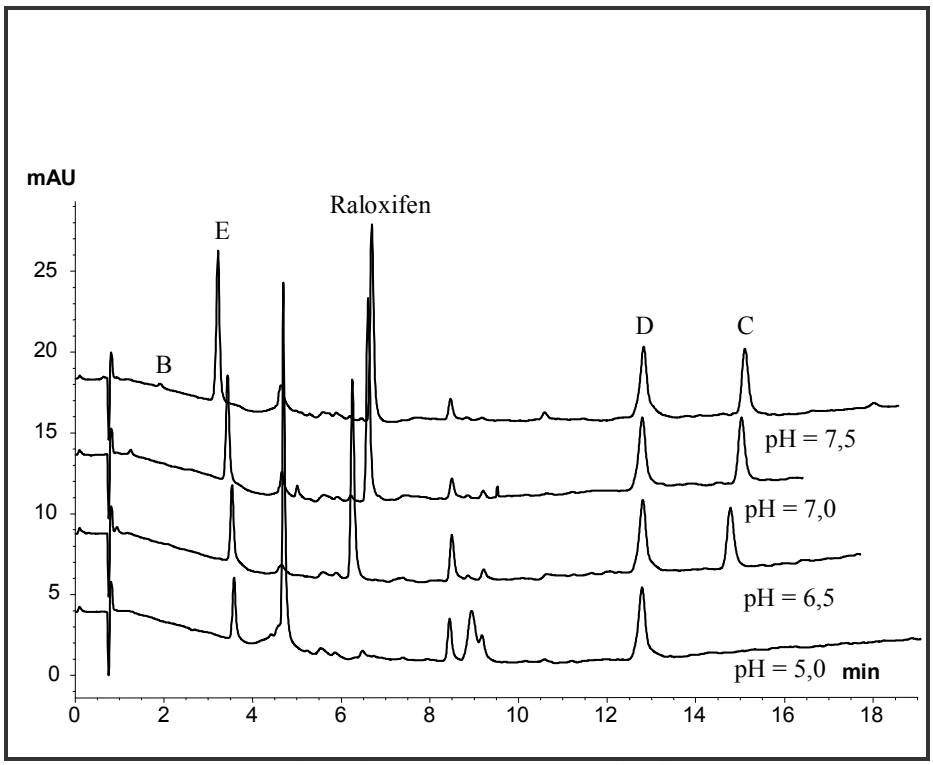

Fig. 2: *Retention of Raloxifen and its related impurities for different pH-values of the aqueous component in the mobile phase.*

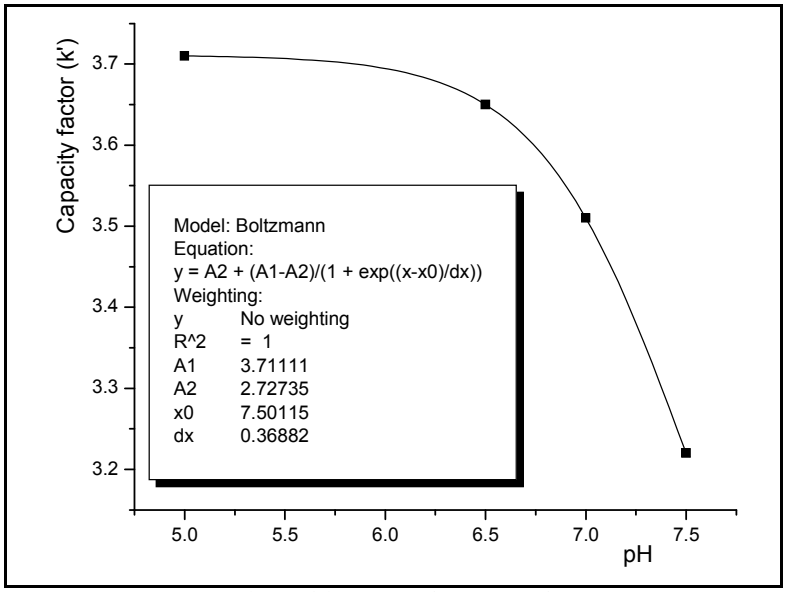

Fig. 3: *Dependence of the capacity factor on pH for impurity E.*

Hidrophobicity parameter

The capacity factor is related to the octanol/water partition coefficient (known as the hydrophobicity parameter, and denoted here by $K_{o,w}$ [5].

$$k'_{ROH} = \beta \cdot K_{o,w}^{ROH} \quad (6)$$

$$k'_{RO^-} = \beta \cdot K_{o,w}^{RO^-} \quad (7)$$

β represents the stationary phase/mobile phase volume ratio in the column. The value of log $K_{o,w}$ can be measured experimentally, by means of shake-flask extraction experiments, or can be theoretically estimated by means of the fragment methodology [6]. This parameter plays a major role in predicting the retention behaviour of analytes in sample in reversed-phase or ion-pair mechanisms [7]. According to this parameter the analytes elute in order given by the increased value of the hydrophobicity parameter.

Organic modifier content in the mobile phase

A polynomial correlation between the capacity factor and concentration of methanol (used as organic modifier) in the mobile phase was observed for these analytes, under gradient elution (with the elution programs given Table 2). Because of different hydrophobicity parameters of the considered analytes, gradient elution conditions were thus necessary. Studies in such conditions have been recently reported and used in estimating different solute properties [9]. The capacity factor (k') for each chromatographic separation was computed, and the dependence of k' on the initial methanol concentration (C_m) in the mobile phase was studied, according to the following linear relationship:

$$k' = A + B_1 \cdot C_m + B_2 \cdot C_m^2 \quad (8)$$

where A can be used in estimating the value of $k_{o,w}$ extrapolated for pure water in the mobile phase that is related to the hydrophobicity parameter by means of the eqs. 6 and 7. Within narrow interval of organic modifier concentration this dependence becomes a linear or polynomial regression. The dependences of the retention on the organic modifier concentration in the mobile phase according to several elution programs given in Table 2 are depicted in Fig. 4 (overlaid chromatograms) and Fig. 5 (as polynomial regressions). The main regression parameters were calculated and given in Table 3.

Table 2. Several elution programs for studying the retention of analytes of interest.

Time (min.)	Program 1		Program 2		Final program		Program 3		Program 4	
	A	B	A	B	A	B	A	B	A	B
0	70	30	74	26	75	25	76	24	80	20
2.5	55	45	59	41	60	40	61	39	65	35
3.5	30	70	34	66	35	65	36	64	40	60
20	10	90	14	86	15	85	16	84	20	80

These dependences can be used in extrapolating to the variations of the mobile phase within ±0.1% around the value of the organic modifier content in the mobile phase. According to

the studied dependences such variations do not influence significantly the retention time and the resolution values between pair of analytes. Therefore, the method is robust towards this parameter.

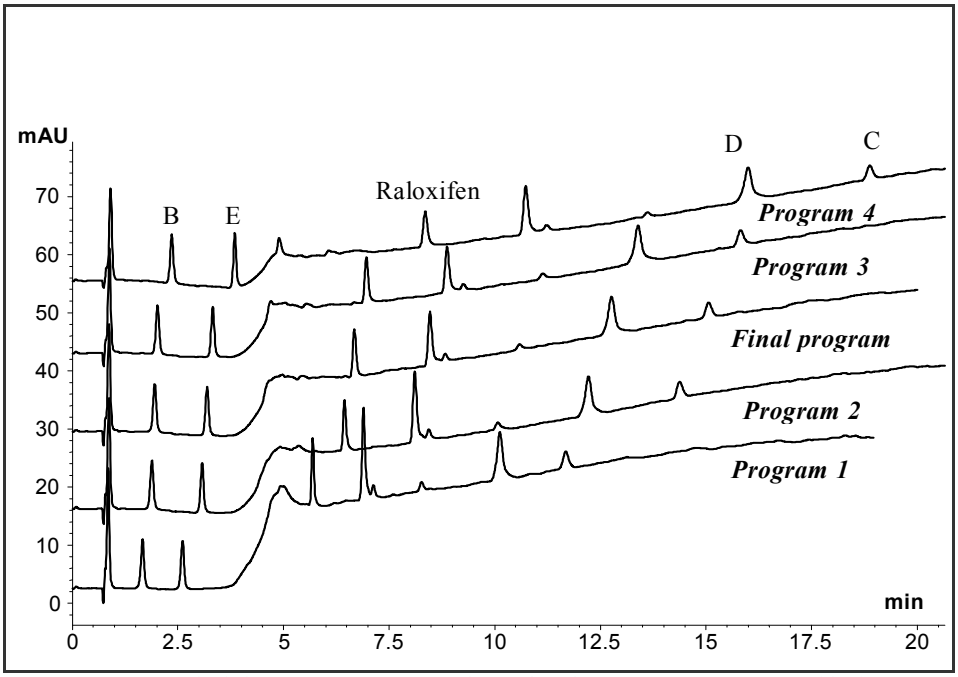

Fig. 4: *Overlaid chromatograms corresponding to five elution programs (given in Table 2).*

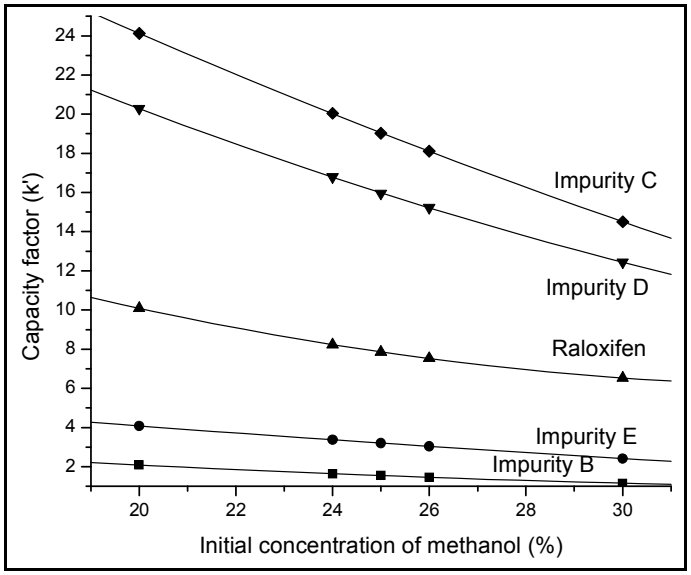

Fig. 5: *Dependences of the capacity factor of Raloxifen and its related impurities on the organic modifier concentration (C) in the mobile phase.*

Table 3. Regression parameters for the dependences given in Fig. 5.

Analyte	A	B_1	B_2	r^2
Raloxifen	27.976	–1.254	0.0180	0.9999
Impurity B	5.869	–0.253	0.0032	0.9996
Impurity C	49.651	–1.487	0.0105	0.9999
Impurity D	44.943	–1.532	0.0149	0.9999
Impurity E	8.477	–0.256	0.0018	0.9999

Temperature

The distribution of an analyte between mobile and stationary phases is governed by the standard free enthalpy (ΔG^0). The relationship between ΔG^0 and the distribution coefficient of the analyte *i* between the two phases (K_i), and finally in terms of the retention data is following:

$$K_i = \beta \cdot k_i' = e^{-\frac{\Delta G^0}{RT}} \tag{9}$$

By changing ΔG^0 with the enthalpy and entropy of transfer of solutes from the mobile phase to the stationary phase, these thermodynamic parameters can be calculated from retention data by evaluation of van't Hoff plots. Thus, the retention factor can be expressed in terms of standard enthalpies and entropies of transfer from the mobile to stationary phase according to the following relationship [10,11]:

$$\ln k' = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \beta \tag{10}$$

The enthalpy (ΔH^0) refers to the transfer of the analyte from the mobile phase to the stationary phase. Entropy (ΔS^0) represents the entropy change of the system (between the mobile and stationary phase). R is the gas constant (8.314 $J \cdot K^{-1} \cdot mole^{-1}$). A plot of ln*k*' vs. $1/T$ (known as van't Hoff plot) is linear, if ΔH^0 and ΔS^0 are independent on the temperature. The slope of the van't Hoff plot gives the standard enthalpies of transfer; the standard entropies of transfer are calculated from the intercept and depend on the phase ratio. The plots of ln *k*' vs $1/T$ in the temperature interval of 20 – 30°C for Raloxifen and its related compounds are given in Fig. 6. Then ΔH^0 and ΔS^0 were estimated by means of the regression parameters and given in Table 4. lnβ was taken as 0.405, considering that usually the porosity volume of a chromatographic column represents approximately 0.4 from the entire volume [12].

These values of ΔH^0 and ΔS^0 are in very good agreement with those obtained for other solutes on similar columns and for the same temperature interval, e.g. aromatic hydrocarbons [13], phenols [14] or basic compounds [15]. Unlike other thermodynamic studies, carried out by similar LC separations, such as for amiodarone and its metabolites [16], this study pointed out quite a different behaviour of one of the compounds (impurity B) in comparison with the others. The difference between the impurity B and the other analytes of interest is that the first one has no ionisable functional groups in order to be

influenced by the *p*H-value of the mobile phase, which at its turn is influenced by column temperature.

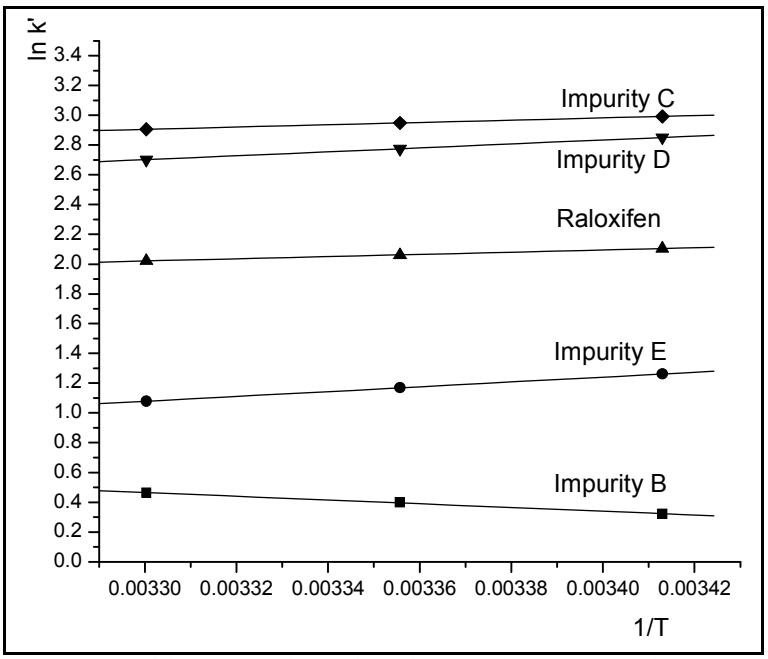

Fig. 6: *Dependence of the retention of Raloxifen and its related impurities on column temperature.*

Table 4: Regression parameters for Raloxifen and its related compounds for the dependence of the retention on temperature and the main thermodynamic parameters.

Analyte	Regression parameters		Thermodynamic parameters	
	a	*b*	ΔH^0 (kJ·mol^{-1})	ΔS^0 (J·mol^{-1}·K^{-1})
Raloxifen	–0.435	744.24	–6.188	–6.98
Impurity B	4.617	–1258.0	+10.459	+35.02
Impurity C	0.357	772.06	–6.419	–0.40
Impurity D	–1.654	1319.98	–10.974	–17.12
Impurity E	–4.279	1623.56	–13.498	–39.94

On the other hand, the regression parameters *a* and *b* for the dependences of the capacity factor for each analyte on column temperature can be used in estimating the variation of their retention time values when column temperature varies within a range of ±0.5°C around 25°C as expected to occur for a validated LC system. In this case, such variations of the column temperature will result in the retention time variations situated within the normal interval of variation of this parameter.

Conclusions

The main parameters influencing the retention of Raloxifen and its related impurities in reversed-phase liquid chromatography were studied and used for developing a chromatographic method for their separation and determination. The dependences can be used in predicting the variation of the retention parameters (retention time, capacity factor, resolution between pairs of analytes) when the chromatographic parameters change randomly their values within a certain interval of variation. On the other hand, the study can be useful in understanding the partition process in reversed-phase liquid chromatography.

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