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A study of some practical aspects of high temperature liquid chromatography in pharmaceutical applications

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ABSTRACT

In the pharmaceutical industry fast and efficient separation techniques play an increasing role among analytical methods because the samples to be investigated grow both in complexity and number, and there is an increasing time pressure to complete the analysis. Reducing the analysis time without decreasing the efficiency is possible using higher pressures, elevated temperatures, smaller particle sizes, or a combination of these approaches. Recently developed chromatographic techniques such as the UHPLC (ultra high performance liquid chromatography) and HTLC (high temperature liquid chromatography) are highly promising in meeting these demands.

In this study, high temperature liquid chromatography (HTLC) with a zirconia-based column and temperatures elevated up to 150 °C was used. We investigated the chromatographic behaviour of a steroid active pharmaceutical ingredient (levonorgestrel) and its structurally related impurities as model compounds. The effect of the temperature in the range of 50–150 °C and the flow-rate in the range of 0.5–3.0 ml/min, and using methanol as an organic modifier, were studied for optimisation of the separation method.

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1. Introduction

HPLC analyses are usually carried out at ambient or slightly above ambient temperature. When using higher temperature, it is possible to improve the analysis conditions, because some physical parameters such as viscosity, mobile phase polarity or diffusivity depend strongly on the temperature. The term "HTLC" is known since 1969, when the Maggs studied the effect of the temperature for small molecules [1] and Antia et al. for large molecules [2]. Nevertheless up until quite recently, elevated temperature has not been considered as a key parameter in LC, and its variation does not appear very often in routine work.

"Elevated temperature" is not an exactly defined term. Different and vague definitions, such as "higher than room temperature", "higher than the boiling point of the mobile phase solvent", and "higher than 100 °C", can be found in the literature [3].

Generally HTLC has had limited use due to the limited temperature range of the thermostat and column in typical HPLC instruments, as well as because of the thermal instability of some of the analytes. There are only two commercially available instruments allowing temperatures to be raised up to 200 °C, which have been specifically optimised for HTLC operation with mobile phase preheating to eliminate thermal mismatch [4], namely the isothermal Metalox® 200C (ZirChrom Separation, Anoka, MN, USA) and the temperature programmable Polaratherm™ Series 9000 (Selerity, Salt Lake City, UT, USA). It is important to note that efficient preheating of the mobile phase is mandatory when operating columns up to 80 °C. Vanhoenacker et al. described the importance of the preheater. Without preheating, the radial centre of the column is at a lower temperature than the incoming cold mobile phase, and the resulting temperature gradient generates viscosity and retention time differences [5]. Fields et al. described the importance of the preheater coil size at different flow rates and came to the conclusion that a longer preheating coil size results in a better peak shape. They drew attention to the importance of other conditions that can affect the peak shapes, such as the injected volume and potentially the composition of the injected solution [6]. Teutenberg et al. have shown that the temperature difference between the eluent temperature and the temperature of the heating block is less than 1 °C at a flow rate of 2 ml/min in a heating system with eluent preheating [7]. The mobile phase cooling after the column is required when using a UV detector in order to avoid baseline noise and to maintain flow cell longevity [8].

The major drawback of the heated column is the risk of stationary phase degradation leading to difficulties when using thermally unstable classical bonded silicas. The development of a new generation of silica based column [9,10] as well as non-silica based ones, has resulted in increased thermal stability. Several station-

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ary phases can be used at elevated temperatures, such as columns based on polystyrene–divinylbenzene, graphitic carbon and ultra-stable metal oxides [11,12] such as zirconia. Polymeric stationary phases can be used up to temperatures of 150 °C, and graphitic carbon columns remain stable up to at least 200 °C. Zirconia-based stationary phases have been shown to be stable at even higher temperatures (200–250 °C) [13].

Presently there is a lack of information about the long-term thermal stability of the different chromatographic supports, moreover there is no universal test which could provide an objective comparison. Many stability studies on various stationary phases have been reported [14–16]. It should be noted that good thermal stability under a given condition is not a guarantee for obtaining long-term stability under different conditions. Although zirconia-based stationary phases are routinely used isothermally at temperatures up to 200 °C, they are not good candidates for temperature programmed high-temperature LC, as opposed to some other columns, such as graphitic carbon and special silica columns [15].

Elevated temperature can decrease the partition coefficient and reduce the retention of a particular analyte, decrease the viscosity of the mobile phase, increase the analyte diffusivity [17]. The consequence of reduced viscosity is a lowered back-pressure over the column, allowing higher speed, the possibility to use longer columns [18] or smaller particle size with higher plate numbers. The consequence of increased diffusivity is increased mass transfer, usually resulting in improved column efficiency. Another advantage of elevated temperature is that the proportion of organic modifier in the mobile phase can be reduced and in some cases eluent containing only water can be used. This technique has been referred to as superheated water chromatography (SHWC), pressurized hot water liquid chromatography (PHW-LC) or subcritical water chromatography (SBWC), and has recently been reviewed by Smith [19].

The use of elevated temperatures to facilitate LC separation is mainly restricted to scientific research and has not been widely applied in routine work. Vanhoenacker et al. [20] investigated the analysis of octylphenol ethoxylates on different stationary phases at low (ambient), medium, and high temperature. They found that the elution order of the oligomers was reversed when comparing ambient and high temperature separation when using acetonitrile/water as the mobile phase. When a protic solvent such as methanol is used, the elution order is reversed compared to acetonitrile at ambient temperature and no reversal as a function of temperature takes place. Sanagi et al. used polybutadiene-coated zirconia stationary phase [21] and carbon-clad zirconia stationary phase [22] for the separation of triazole fungicides. The separation of free sterols by high temperature LC was tested by Riddle and Guiochon [23]. The graphitic carbon column studied provides the best separation factors. The observed effects include selectivity improvements and elution order reversal. The analysis of some steroids was shown by Fields et al. [6] using superheated water as eluent on a polymer-coated zirconia column. Al-Khateeb et al. used a hybrid stationary phase for the high temperature chromatography of hydrophobic steroids [24] and studied the effect of temperature (up to 130 °C) on the retention and the efficiency. They concluded that the proportion of the organic solvent for elution can be reduced at elevated temperatures for the non-polar analytes, too, and superheated water can be used as eluent.

In the present paper, results concerning the chromatographic behaviour of a steroid active pharmaceutical ingredient (levonorgestrel) and some of its related impurities is shown on zirconia-based column at elevated temperatures. In addition, the effects of varying the organic modifier and the flow-rates are also reported. Our results and the latest theoretical considerations are compared [27].

2. Experimental

2.1. Chemicals and samples

The separations were performed on a Discovery Zr-CarbonC18 column (Zr-CarbonC18) 3.5 μm, 150 mm × 4.6 mm produced by Supelco (Bellefonte, USA). Eluents were prepared from HPLC grade methanol (Merck, Germany) and water in different ratios and used at a flow-rate range of 0.5–3.0 ml/min. Water was purified by Milli-Q Water Purification System (Millipore, USA) equipped with a 0.22 μm filter. A model solution of levonorgestrel and its impurities were used in the concentration of 0.01 mg/ml, each, dissolving them in methanol:water = 1:1.

Steroids tested were prepared at Chemical Works of Gedeon Richter Plc. (Budapest, Hungary) and were of the highest available quality. Their structures are shown in Fig. 1.

2.2. Instrument

An Agilent 1100 HPLC (Waldbronn, Germany) system equipped with a diode array detector was employed at 244 nm. All analyses were carried out in an isocratic mode. The column temperature was controlled by a Polaratherm Series 9000 (Salt Lake City, USA) oven equipped with a mobile phase preheater in the range of 50–150 °C with a precision of ±1 °C. The preheater temperature was set equal to the oven temperature and the effluent temperature was 40 °C. The chromatograms were processed using Agilent ChemStation (Rev.A.10.02.) software and ChemStore (Rev.B.02.02) database. The data were handled by Origin 7.5 software.

3. Theory

3.1. Dependence of the column efficiency on temperature

The value of linear velocity (u) can be calculated according to

$$u = \frac{F_v}{\pi R_c^2 \varepsilon_T}, \quad (1)$$

where F_v is the flow-rate (ml/min), R_c is the radius of the column (cm) and ε_T the total porosity of the column [28].

The plate height of a column can be optimised by varying the linear velocity, u of the mobile phase as described by the van Deemter equation:

$$H = A + \frac{B}{u} + Cu, \quad (2)$$

where A is the eddy diffusion term, B/u is the longitudinal diffusion term, and Cu is the mass transfer term [1]. Theoretically, the effect of temperature on the A term is uncertain, but it is expected that elevated temperatures will improve the laminar flow and lateral mixing of the analyte molecules from different flow channels due to the increased diffusivity, although the improvements may not be significant [25]. The minimum plate height is independent of temperature for fast kinetics, but the minimum plate height decreases with increasing temperature in systems with slow kinetics [2]. The coefficient A, which is a measure of how well the column is packed, is normally little affected. The longitudinal diffusion term B, which reflects the geometry of the eluent in the column, increases with increasing temperatures, and becomes significant at low linear velocities. The C term, which represents the mass transport between phases and diffusion inside the stationary phase as well as the adsorption/desorption kinetics, decrease with increasing temperatures. Therefore the minimum point of the van Deemter curve shifts towards higher linear velocities with increasing temperatures.

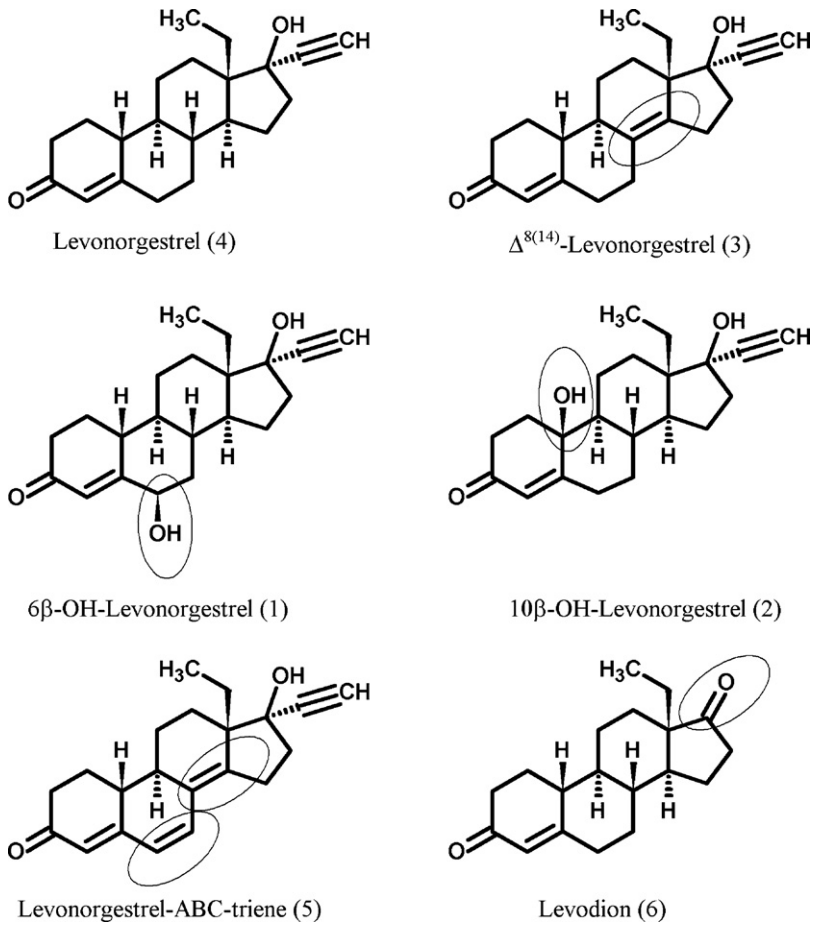

Fig. 1. Structures of levonorgestrel and related compounds.

3.2. Effect of the temperature on the separation

The effect of temperature on the retention factor, k, can be described using the van't Hoff equation,

$$\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \Phi, \quad (3)$$

where ΔH° is the enthalpy of transfer from the stationary to the mobile phase, ΔS° is the associated change in standard entropy, and Φ is the phase ratio of the column [1].

In the limit of ΔH°, ΔS° and Φ being invariant with temperature in Eq. (3), a plot of $\ln k$ versus $1/T$ gives a straight line with a slope of $-(\Delta H^\circ/R)$ and an intercept of $\Delta S^\circ/R + \ln \Phi$. Usually this linearity proves to be a very good practical approximation for neutral compounds. However, there are cases when the curves deviate from linearity, which can sometimes be treated in terms of dividing the curve into two linear plots which intersect at the transition temperature [26]. Guillarme et al. [27] observed a dependence of the solute behaviour on the type of solvents. The van't Hoff plots were linear for water–methanol mixtures while curved in the case of water–acetonitrile when using organic polymer as the stationary phase.

4. Results and discussion

In our work the separation of levonorgestrel and some of its impurities were studied on Zr-CarbonC18 column at elevated temperatures. The initial separation was run at 50 °C using 60% MeOH in water as the eluent at a flow rate of 0.5 ml/min. The chromatogram is shown in Fig. 2.

4.1. Relationship between retention and structure

The dependence of retention on temperature was studied over the temperature range of 50–150 °C (Fig. 3) for levonorgestrel and its impurities. 150 °C constituted an upper temperature limit because of the thermal instability of the steroid compounds investigated. A systematic decrease was observed in the retention factor with increasing temperature. The structural differences of the compounds are highlighted in Fig. 1. The curves of $\ln k$ versus $1/T$ are

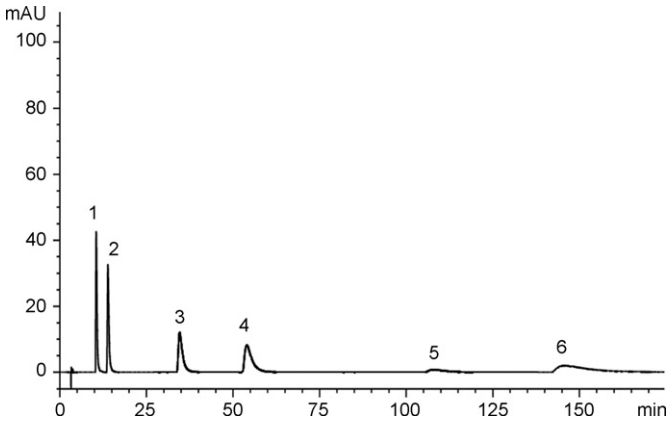

Fig. 2. Separation of levonorgestrel and its related compounds under the initial chromatographic conditions: column Discovery Zr-CarbonC18 (150 mm × 4.6 mm, 3.5 μm); temperature 50 °C; eluent 60% methanol in water; flow-rate 0.5 ml/min; injection 20 μl of a model solution of levonorgestrel in the concentration of 0.01 mg/ml; detection at 244 nm and the numbering see in Fig. 1.

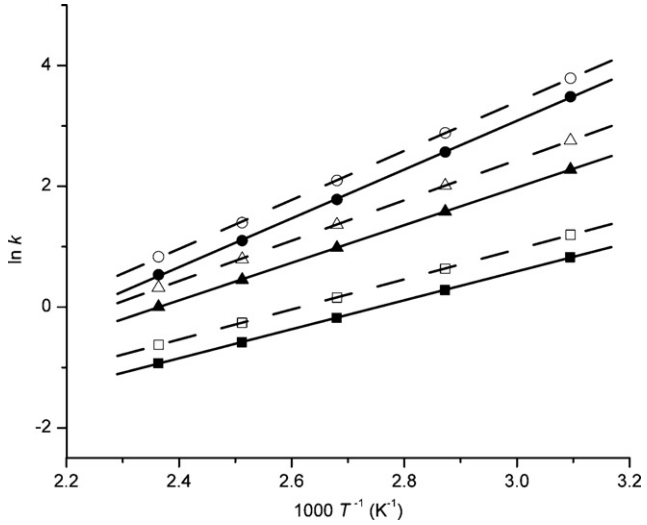

Fig. 3. The ln k versus $1/T$ curves for each steroid component. The components are 6β-OH-levonorgestrel (■), 10β-OH-levonorgestrel (□), $\Delta^{8(14)}$-levonorgestrel (▲), levonorgestrel (△), levonorgestrel-ABC-triene (●), and levodion (○). Conditions: temperature range 50–150 °C; other parameters see in Fig. 2. The linear equation for levonorgestrel is $\ln k = 3341.98\,T^{-1} - 7.59$, $R^2 = 1.000$

Table 1
The change of the enthalpy for each component calculated by the van't Hoff equation.

Name of component	ΔH° [kJ mol^{-1}]
6β-OH-levonorgestrel	20.0
10β-OH-levonorgestrel	20.7
$\Delta^{8(14)}$-Levonorgestrel	25.9
Levonorgestrel	27.8
Levonorgestrel-ABC-triene	33.6
Levodion	33.8

linear with correlation coefficients greater than 0.99 at a flow rate of 0.5 ml/min and the slopes of the linear curves and the calculated changes of the enthalpy are almost equal to the structurally similar components, which are presented in Fig. 3 and Table 1.

The relationship between the temperature and the retention time for levonorgestrel and its impurities being very similar, the other parameters of optimisation (organic modifier content and flow rate) were investigated only for levonorgestrel as the main peak.

To reduce the lengthy analysis time and to study the behaviour of levonorgestrel and its impurities the following optimisation steps were investigated.

4.2. Effect of the temperature on separation efficiency

The first optimisation parameter was the temperature in the range of 100–150 °C.

The measured dependence of the plate height (H) on the linear velocity (u) at different temperatures was plotted by the van Deemter equation as shown in Fig. 4.

The actual data, represented by markers, were fitted using the van Deemter equation. The optimum linear velocity is increasing with increasing temperatures. The temperature effects on the A, B and C terms were calculated and the results for the C term are listed in Table 2.

It can be seen that the C term significantly decreases by increasing the temperature. The diffusion coefficient of the solute in the mobile phase is the main reason for the decrease in the C term, which facilitates faster and more efficient separations.

Selecting the best conditions the 150 °C temperature was used for further studies.

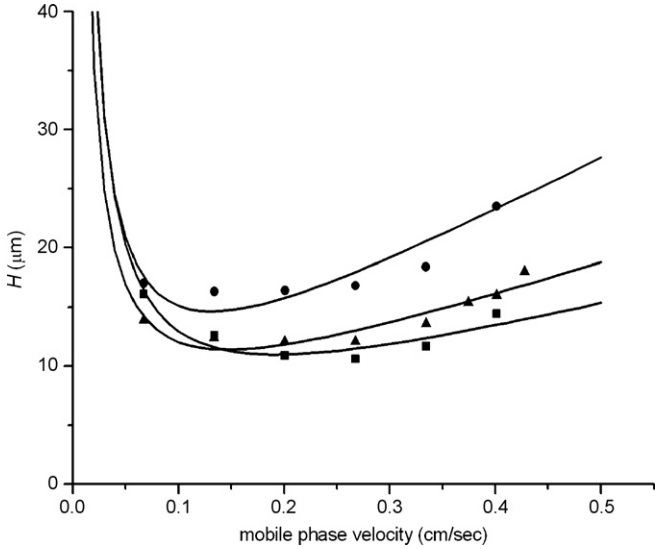

Fig. 4. Dependence of plate height (H) on mobile phase velocity (u) applying different temperatures for levonorgestrel: (●) 100 °C, (▲) 125 °C, and (■) 150 °C. Conditions: eluent 60% methanol in water; flow-rate range 0.5–3.0 ml/min; other parameters see in Fig. 2.

Table 2
Effect of temperature on the C terms calculated by the van Deemter equation.

t [°C]	C [10^3 s^{-1}]
100	0.48
125	0.30
150	0.23

4.3. Effect of the methanol content of the eluent on separation efficiency

When the solvent strength (% MeOH) is varied in isocratic elution, solute retention is related to the volume fraction of the organic solvent as

$$\log k = \log k_w - S\Phi. \quad (4)$$

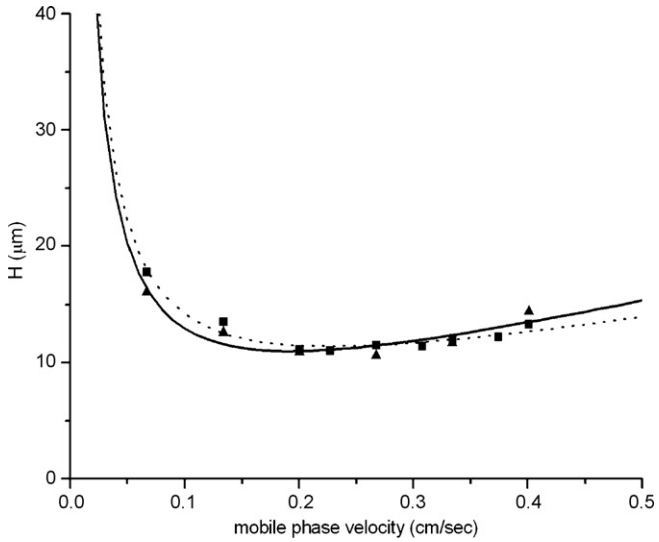

Fig. 5. Plots of plate height (H) versus mobile phase velocity (u) for levonorgestrel at different mixture of methanol and water: (■) 60% methanol, and (▲) 50% methanol. Conditions: temperature 150 °C; flow-rate range 0.5–3.0 ml/min; other parameters see in Fig. 2.

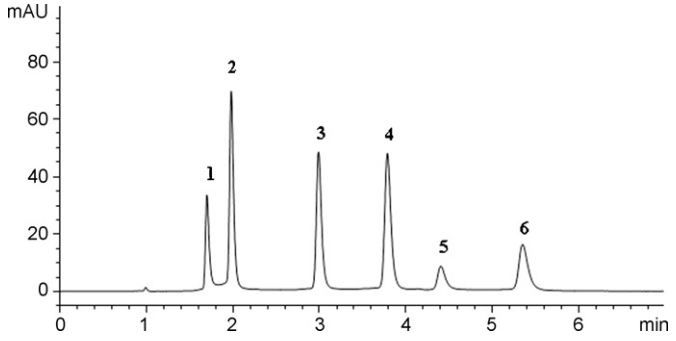

Fig. 6. Fast and selective separation of levonorgestrel and its related compounds. Conditions: temperature 150 °C; eluent 50% methanol in water; flow-rate 1.5 ml/min; other parameters see in Fig. 2 and the numbering see in Fig. 1. Resolutions: $Rs_{1,2}$ = 3.5, $Rs_{2,3}$ = 10.5, $Rs_{3,4}$ = 6.6, $Rs_{4,5}$ = 4.2, and $Rs_{5,6}$ = 5.3.

As seen in Eq. (4) the retention decreases with increasing organic solvent content. Fig. 5 shows that there is no significant difference between the van Deemter curves at different percentages of methanol (50% and 60%), therefore the lower methanol content in the eluent was used as the mobile phase in the next optimisation steps.

4.4. Effect of the flow rates on separation efficiency

Fig. 5 shows the dependence of the plate height on the linear mobile phase velocity in the flow-rate range of 0.5–3.0 ml/min. The minimum plate height can be achieved in the flow-rate range of 1.5–2.0 ml/min at 150 °C, 50% MeOH.

Summarising our experiments for levonorgestrel and its structurally related compounds the presently optimised chromatographic parameters are as follows: temperature 150 °C, methanol content 50% in water, and flow rate 1.5 ml/min. Because of the proper resolutions between the critical peak pairs (Fig. 6), this improved method may be implemented in the routine purity testing of levonorgestrel API.

5. Conclusion

Using a steroid active pharmaceutical ingredient and its related impurities as model compounds we have studied the effects of elevated temperatures on their HPLC separation with a look to improving chromatographic efficiency. To that end, a series of experiments were designed to better understand the practical aspects of increasing temperatures. For the separation of the model compounds a new generation zirconia column and methanol–water eluent were used. Our results indicate that the traditional chromatographic equations can be expanded for elevated temperatures as well. The elution order of these types of steroids on zirconia-based stationary phase was the same as on silica-based RP columns. Optimised separation parameters resulted in an improved method to separate levonorgestrel and its related impurities in very short analysis times, and this may be applied for routine analytical investigations. As a continuation of this work, further optimisation steps and a comparison of the best HTLC methods to other HPLC, TLC, HPTLC or OPLC techniques are planned.

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