Separation of Steroids by Temperature-Programmed Capillary Liquid Chromatography Using Packed Fused-Silica Column

Separação de Esteróides por Cromatografia Líquida Capilar Programada por Temperatura Usando Coluna de Sílica Fundida Empacotada

Abstract
The miniaturization of liquid chromatography and the development of fused silica capillary packed columns have enable a fast and homogeneous heat transmission through the analytical column. Thus, the use of temperature programming instead of gradient of mobile phase becomes an alternative to decrease the analysis time. This manuscript reports on the applicability of an analytical method for the separation of five steroids by temperature programming capillary liquid chromatography. The influence of the temperature on analytes retention time was investigated and discussed. The steroids were separated by a lab-made capillary column (RP-18) under isocratic elution. The temperature programming allowed to reduce twice the analysis time and diminished the band broadening of the most retained compound in comparison with isothermal analysis.

Keywords: capillary liquid chromatography, temperature programming, steroids.

Resumo
A miniaturização da cromatografia líquida e o desenvolvimento de colunas capilares de sílica fundida empacotadas com a fase estacionária possibilitaram a rápida e homogênea transferência de calor na coluna analítica. Desta forma, o uso da programação de temperatura ao invés do gradiente de fase móvel torna-se uma alternativa para diminuir o tempo de análise. Este trabalho relata a aplicabilidade de um método analítico para a separação de cinco esteróides utilizando cromatografia líquida capilar com programação de temperatura. A influência da temperatura na retenção dos analitos foi investigada e discutida. Os esteróides foram separados uma coluna capilar lab-made (RP-18) utilizando eluição isocrática. A programação de temperatura permitiu a redução do tempo de análise em duas vezes e diminuiu a dispersão da banda cromatográfica do composto mais retido em comparação com análises em condições isotérmicas.

Palavras-chave: cromatografia líquida capilar, programação de temperatura, esteróides.
1. Introduction

The use of columns with reduced internal diameter in liquid chromatography (LC) has several advantages over the conventional LC column, such as low consumption of mobile phase, low sample volume injection and possibility of applying temperature programming (TP) during the analyses\textsuperscript{1-4}. Due to the large bore of the standard LC columns (4.0 – 4.6 mm i.d.) the radial temperature gradient creates difficulties for the accomplishment of TP in such a column. However, the development of capillary LC columns using fused silica tubes whose internal diameter is equal or smaller than 0.25 mm has enabled a fast and homogeneous heat transmission through the column wall.

The first report on the effect of temperature on chromatographic retention was published in 1946\textsuperscript{5}, although TP in LC was reported only in 1966\textsuperscript{6}. The study of the effect of temperature on miniaturized LC (both microbore and capillary LC) began in the 1980’s\textsuperscript{7} with extensive researchers lidered by Ishii, Sumiya, Jinno and McNair groups. Thompson and Carr\textsuperscript{8} claimed that the stability of the analytes should not be affected when the compounds were exposed to high temperatures for a relatively short time. The thermal stability of the stationary phases used in LC has been discussed in the literature\textsuperscript{9,10}. Some papers and reviews describing the applicability of high temperature or TP for separations in LC have been reported\textsuperscript{11-15}.

In general, the effects on the analytes retention time produced by TP are similar to those observed when solvent gradient is applied. The dependence of the dielectric constant of water (\(k_{\text{water}}\)) as a function of temperature can be approximated by the Equation 1:

\[
k_{\text{water}} \approx 80 - 0.4 (T - 273)
\]  \hspace{1cm} (1)

where \(T\) is the absolute temperature (Kelvin). The Equation 1 demonstrates that increasing the analysis temperature reduces the water polarity. Thus, at elevated temperature, the aqueous solvent shows characteristics comparable with organic polar solvent (methanol or acetonitrile).

Another consequence of increased analysis temperature is the lowered viscosity of the mobile phase according to Equation 2\textsuperscript{16}.

\[
\eta = 10^{a + b T^c}
\]  \hspace{1cm} (2)

where \(\eta\) is the viscosity, \(T\) is the absolute temperature (Kelvin), and \(a\), \(b\), and \(c\) are coefficients that are specific to a solvent.

The mobile phase viscosity is directly proportional to pressure drop (\(\eta \propto \Delta P\)) and increasing the temperature, the pressure of the chromatographic system is diminished.

Therefore, the use of temperature programming in capillary packed liquid chromatography (TP-cLC) is considered advantageous for the reduction in the analysis time when the solvent gradient causes negative impacts on the detection (baseline variation on UV monitoring, changes in MS ionization using ESI) or when the high temperature of the eluent increases the sensitivity (ELSD). If the mobile phase is free of organic solvent, a low wavelength UV or a FID detector can be used with HPLC\textsuperscript{17}.

Steroids comprise an important class of therapeutic compounds of similar chemical structure consisting of 17 carbon atoms arranged in the form of four interconnected rings. Among the methods developed to monitor steroids, a large number of papers employing HPLC have been published. These methods use mobile phase gradient or high temperature approach for steroids separation\textsuperscript{18}. However, decreasing the analysis time without diminishing the resolution may be more effective when the gradient temperature is used during the chromatographic assay.

This paper reports on the discussion and applicability of TP-cLC for separation of estriol, estrone, progesterone, cortisone acetate and hydrocortisone acetate with no mobile phase gradient.
2. Experimental

2.1. Materials and reagents

Analytical standards of estriol, estrone, progesterone and cortisone acetate were supplied by Sigma-Aldrich (St. Louis, USA) and hydrocortisone acetate was obtained from Alfa Aesar (Ward Hill, USA). HPLC-grade acetonitrile (ACN) was purchased from J. T. Baker (Phillipsburg, USA) and high-purity water was obtained from an Elga purification system (High Wycombe, UK). Stock solutions containing 500 mg L\(^{-1}\) of steroids were prepared dissolving each of the analytes in ACN:H\(_2\)O (60:40, v/v). Subsequently, a working solution containing the five steroids at a concentration of 50 mg L\(^{-1}\) was prepared by diluting the stock solutions.

2.2. Chromatographic System

The analyses were performed in a chromatographic system composed of a homemade high-pressure pump and a micro-oven with high precision temperature control\(^{[19]}\). The sample was injected by a Valco valve (Houston, USA) of 60 nL internal loop volume connected to an electric actuator. The detection was achieved by an SPD-M10A VP Photodiode Array Detector connected to a CBM-20AD controller, in compliance with data acquisition software LabSolutions-LCsolution from Shimadzu (Kyoto, Japan). A capillary U-shaped detector cell with 8 mm light path and 35 nL (UZ-SHM-CAP) was purchased from LC Packings (Amsterdam, the Netherlands) and used in the experiments.

2.3. Preparation of the capillary column

A capillary column (150 mm x 250 μm i.d.) containing Kromasil RP-18 silica with 3.5 μm (Eka Chemicals, Bohus, Sweden) was packed in our laboratory through the slurry packing technique and according to a procedure previously described in the literature\(^{[20]}\). Frit of approximately 0.2 mm length was placed inside capillary by punching its tip against a glass fiber filter (Whatman GF/A, W & R Balston, UK). A 50 μm i.d. and 150 mm capillary was used to insert and retain the glass fiber frit approximately 5 mm from the column tip. Epoxy resin (Araldite 10 minutes, Brascola, Sao Paulo, Brazil) was used to keep the junction between the column capillary tubing and the connecting tubing. Slurry was composed of 8.0 mg of microparticles in a mixture containing isopropanol:tetrahydrofuran (1:6, v/v). It was sonicated for 10 minutes and placed in a stainless steel reservoir (3.5 mL) connected to the column capillary tubing. A Shimadzu pump (model LC-20AD, Kyoto, Japan) operating at 10 MPa was used to delivery the packing solvent (MeOH) from the reservoir. When the packed bed have been achieved, the pumping process was stopped and the column was slowly depressurized to ensure minimal movement of the particles. The capillary column was connected to the chromatographic system by zero-dead-volume unions (Upchurch Scientific, Oak Harbor, WA, USA) and conditioned for two hours with ACN:H\(_2\)O (70:30, v/v) before the use.

3. Results and discussion

The mobile phase used for the steroids separation was previously optimized by ACN:H\(_2\)O in three different proportions under isothermal conditions (Figure 1). As a result, the mobile phase containing ACN:H\(_2\)O (40:60, v/v) showed the best resolution between hydrocortisone and cortisone (Rs\(_{2,3}\) = 2.73) with a long analysis time (50 minutes). Then, a study using temperatures of 30°C, 60°C and 80°C under isothermal condition were performed to evaluated the influence of temperature in the chromatographic parameters of steroids (Figures 2A-C).

The influence of temperature on chromatographic retention can be expressed by van’t Hoff equation (Equation 3).

\[
\ln k = -\frac{\Delta H^\circ}{RT} + \left(\frac{\Delta S^\circ}{R}\right) + \ln \phi
\]

(3)

where \(k\) is the retention factor; \(\Delta H^\circ\) is the system enthalpy; \(\Delta S^\circ\) is the system entropy; \(T\) is the absolut temperature (Kelvin); \(R\) is the gas constant (8.31441 J K\(^{-1}\) mol\(^{-1}\)); and \(\phi\) is the volume ratio of stationary to mobile phase.
This equation describes the contribution of thermodynamics parameters on retention of analytes. This relationship shows that for a given temperature variation, the retention factor for analytes with higher enthalpy is more affected than analytes with lower enthalpy values. Thus, a change in temperature can provide different selectivity and promoted the separation of analytes\cite{21}.

The Figure 3 shows the van’t Hoff plot \(1/T\) plotted against the \(\ln k\) that express the relationship between the temperature and retention factor. A positive linear slope under reverse phase conditions was observed for all the steroids and suggests that (1) the retention mechanisms are constant over the temperature range under consideration and (2) an increase in temperature causes a decrease in retention factor.

When the column chromatography was kept at 30°C (Figure 2A), the five steroids were completely separated, but as illustrated, the last eluting peak (progesterone, \(k = 23.59\)) were severely broadened and the analysis time was long (50 minutes). At 60°C (Figure 2B), the analysis time had been considerably reduced to 35 minutes with no overlapping of the chromatographic peaks (\(R_{s,3} = 1.90\)). However, when the column temperature was set to 80°C (Figure 2C), the analysis time was decreased (progesterone showed...
however it causes loss of the resolution and selectivity for hydrocortisone acetate and cortisoline ($R_s = 1.25$ and $\alpha = 1.15$). According to resolution master equation, the three terms in the equation (retention factor, selectivity and efficiency) are interdependent, i.e., small changes in $k$ usually cause changes in $\alpha$, therefore, appreciable changes in resolution are also observed. Since the resolution is increased with increasing values of $N$, $\alpha$ and $k$, any factor that may alter these parameters may improve $R_s$. Change temperature affects all of them and therefore the resolution.

Since the isothermal separation of steroids at 60°C showed good results for hydrocortisone and cortisoline ($R_s = 1.91$ and $\alpha = 1.18$), this temperature was chosen to start the chromatographic analysis by TP-cLC to reduce the analysis time. During the first

Figure 2. Comparative chromatograms obtained under isothermal (A-C) and TP (D) conditions for the analysis of a standard mixture containing 50 mg L$^{-1}$ of steroids. Mobile phase: ACN:H$_2$O (40:60, v/v). Flow rate: 3.0 µL min$^{-1}$. Detection UV: 234 nm. Order of elution: (1) estriol, (2) hydrocortisone acetate, (3) cortisol acetate, (4) estrone and (5) progesterone.
Figure 3. van't Hoff plot of steroids at temperature of 30°C, 60°C and 80°C during RP-HPLC experiments.

Table 1. Chromatographic parameters obtained for steroids separation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$t_r$ (min)</th>
<th>$k$</th>
<th>N/m</th>
<th>$R_s$</th>
<th>$\alpha$</th>
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<tbody>
<tr>
<td><strong>Isothermal - 30°C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Estriol</td>
<td>3.30</td>
<td>0.69</td>
<td>10,048</td>
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<tr>
<td>Hydrocortisone acetate</td>
<td>8.56</td>
<td>3.39</td>
<td>22,453</td>
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<tr>
<td>Cortisone acetate</td>
<td>10.17</td>
<td>4.22</td>
<td>31,693</td>
<td>2.73</td>
<td>1.24</td>
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<tr>
<td>Estrone</td>
<td>15.93</td>
<td>7.17</td>
<td>58,539</td>
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<tr>
<td>Progesterone</td>
<td>47.96</td>
<td>23.59</td>
<td>64,023</td>
<td></td>
<td></td>
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<td><strong>Isothermal - 60°C</strong></td>
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<tr>
<td>Estriol</td>
<td>2.80</td>
<td>0.44</td>
<td>13,380</td>
<td></td>
<td></td>
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<tr>
<td>Hydrocortisone acetate</td>
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<td>2.39</td>
<td>23,015</td>
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<td>Cortisone acetate</td>
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<tr>
<td>Estriol</td>
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<td>0.44</td>
<td>13,380</td>
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<td>Progesterone</td>
<td>25.00</td>
<td>11.82</td>
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<td>Estriol</td>
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<td>Hydrocortisone acetate</td>
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<td>3.95</td>
<td>86,104</td>
<td></td>
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<tr>
<td>Progesterone</td>
<td>20.34</td>
<td>9.43</td>
<td>76,402</td>
<td></td>
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</tbody>
</table>

7 minutes, the temperature was kept at 60°C to avoid for satisfactory resolution between hydrocortisone acetate and cortisone acetate. Subsequently, a temperature gradient of 20°C min$^{-1}$ was applied to reach 100°C and remained isothermal at this temperature until the end of the analysis (Figure 2D). Under such conditions, the duration of the analysis with temperature gradient was 22 minutes ($k = 9.43$ for progesterone), with column efficiency of 86,104 N/m (estrone). A short temperature
equilibrium time of 2 minutes was required in order to reset the initial conditions for next analysis. From the chromatograms, retention time ($t_R$), retention factor ($k$), number of plates ($N/m$), resolution ($R_s$) and selectivity ($\alpha$) were calculated. The results of the chromatographic parameters are summarized in Table 1.

Baseline variation in LC-UV experiments using mobile phase gradient has been often reported and affect the quantification. These effects were not observed during temperature programming analyses and no baseline correction was applied.

In fact, reducing peak band broadening is a good strategy to improve the column efficiency. Thus, the utilization of temperature programming during the chromatographic process may decrease the peak widths and might also affect the peak intensity, especially for longer retained compounds. The comparison between the experiments in Figures 2A and 2D shows that there was an increase of 65 % in peak intensity and a reduction of 60 % in peak width ($w_b$) for progesterone. This approach enables the reduction of the analysis time approximately 50 % and 30 % in comparison with the chromatograms obtained at 30°C and 60°C, respectively, with no decomposition and overlapping peak of steroids.

4. Conclusion
This study has demonstrated that the use TP-cLC for the analysis of steroids is a very attractive alternative for a reduction in analysis time without the use of mobile phase gradient. The steroids were separated performed by TP-cLC with no degradation of both analytes and column during the analysis. The TP-cLC method reduced twice the analysis time in comparison with the chromatogram at 30°C under isothermal condition.

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References


