Theses of the PhD Dissertation

Study on the role of temperature in the analysis of a chiral active pharmaceutical ingredient: application of HPLC and NMR techniques

Renáta Gerencsér-Berta

Supervisor: Dr. Mária Gazdag, PhD

Consultant: Dr. Ibolya Molnár-Perl, Professor Emerita

Eötvös Loránd University

Doctoral School of Chemistry
Head: Dr. György Inzelt, Professor

Analytical Chemistry, Colloid- and Environmental Chemistry, Electrochemistry Program
Head: Dr. Gyula Záray, Professor

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1. Introduction, Aims

Pharmaceutical research and manufacturing poses an increasing demand for the acceleration of processes. Thus, efficient analytical and separation methods are elaborated to answer the problems arising both research and development and the quality assurance of drugs within the shortest possible period of time. Application of rapid chromatographic techniques has gained in importance, including the promising method of high-temperature liquid chromatography (HTLC).

High-temperature liquid chromatography enables the reduction in analysis time by increasing the temperature, hence it lends itself to separate substances that are thermally stable and do not suffer degradation during analysis. Applicability of the method is limited by the heat-resistant characteristics of the instrument and the stationary phase. While silica-based columns exhibit limited chemical and thermal stability, several of the recently introduced new-generation stationary phases (based on graphitic carbon and ultra-stable metal oxide like alumina or zirconia) are applicable within a wider range of temperature. Column thermostat providing a constant temperature is an essential part of instruments dedicated for elevated temperature work, preheating of the mobile phase before reaching the column is important to avoid peak broadening and cooling the solvent leaving the column to the temperature of the detector to minimize baseline noise. The column thermostat can be used as a standalone or a built-in module with optimized parameters in modern instruments.

Theoretical conditions of liquid chromatography remain applicable without modifications in the HTLC technique. Temperature dependence of the retention factor provides information on the thermodynamic parameters of equilibrium processes between mobile and stationary phases in both achiral and chiral systems. However, the separation mechanism in chiral analysis is more complex due to the additional host-guest interaction, hence it is difficult to predict which chiral selector will provide enantioresolution. NMR spectroscopic techniques can be helpful in this regard.

The primary aim of my PhD work was to explore the effect of temperature on the achiral and chiral HPLC analysis of Levonorgestrel, a steroidal active pharmaceutical ingredient (API). Owing to the wide polarity range of its achiral impurities, separation on a silica-based stationary phase has a long analysis time. My object was therefore to select a modern, non-silica-based column specially developed for elevated temperatures, where effects of chromatographic parameters and conditions (temperature, the identity and proportion of the

organic modifier in the mobile phase, flow rate) are to be studied on retention behavior of model compounds.

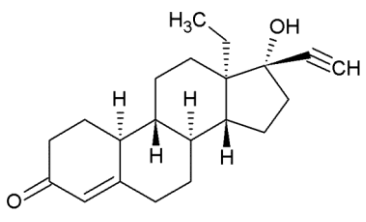
In the case of chiral drug substances such as Levonorgestrel, exact quantitative determination of the eventual enantiomeric impurity represents a necessary and imperative task. Considering literature data and the experience collected previously at Richter, the influence of native cyclodextrins (CDs) applied in the mobile phase was studied during the HPLC separation of Norgestrel enantiomers over an extended temperature range. Permethylated (PM) CD derivatives were also used either in the mobile or the stationary phase in additional experiments.

To gain more insight into the mechanism of chiral separation, we applied NMR spectroscopic methods. Enantiomer-specific stability constants towards native or permethylated CDs were determined by ^{1}H NMR titrations of racemic Norgestrel. The temperature dependence of these equilibrium constants was used as an independent corroboration of the HPLC-based thermodynamic parameters. The stoichiometry of the formed complex(es) was assessed by Job's method, while we made attempts to identify the molecular fragment(s) of the guest mostly involved in the binding from 2D NOESY (ROESY) spectra.

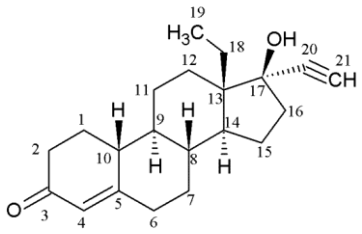
During my doctoral studies, I myself performed the HPLC measurements, their evaluation and conclusions. The NMR measurements, their evaluation and interpretation were conducted in collaboration with Dr. Zoltán Szakács, a colleague at Spectroscopic Research Department. In the following, my own results will be presented in singular form, while the cooperative ones in plural.

2. Methods

The subject of our experiments was Norgestrel, the steroidal API manufactured and marketed for a long time at Gedeon Richter Chemical Works Plc. Chiral studies were conducted on racemic Norgestrel, containing *d*- and *l*-Norgestrel in 1:1 proportion (Figure 1), while the achiral experiments targeted Levonorgestrel and its specially selected impurities (Figure 2).

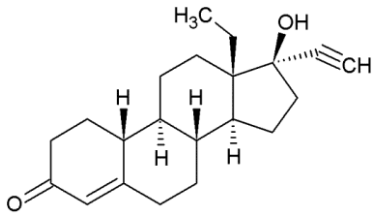


l-Norgestrel

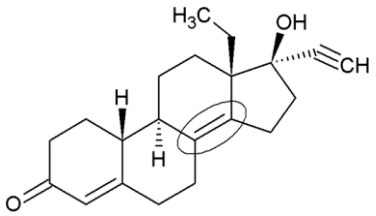


d-Norgestrel
(Levonorgestrel)

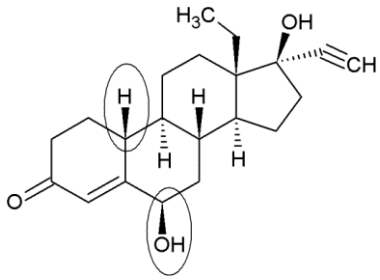
Figure 1. Structural formulae of *d*- and *l*-Norgestrel



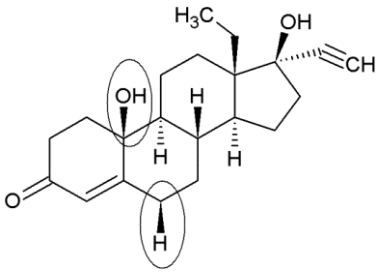
Levonorgestrel



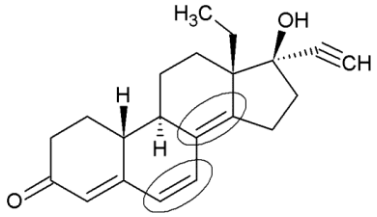
$\Delta^{(8,14)}$-Levonorgestrel



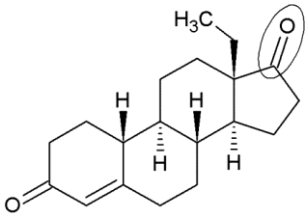
6β-OH-Levonorgestrel



10β-OH-Levonorgestrel



Levonorgestrel-ABC-triene



Levodione

Figure 2. Structural formulae of Levonorgestrel and its impurities, highlighting the structural differences

The solvents and chemicals used were of the best available quality. The following analytical methods were applied:

- I performed the ***achiral HPLC*** runs on an Agilent 1100 HPLC instrument in isocratic mode. A Discovery Zr-CarbonC_{18} stationary phase were used, the constant temperature was controlled by a Polaratherm Series 9000 oven, providing also preheating of the mobile phase (50–150 °C) and its cooling before reaching the detector (40 °C). Different mixtures of methanol-water or acetonitrile-water were applied.

- I conducted the ***chiral HPLC*** studies on an achiral Luna C_{18} (2) or the chiral Nucleodex PM-α-, PM-β- and PM-γ-cyclodextrin stationary phases in methanol-water eluents of different composition using an Agilent 1100 HPLC instrument. Native and permethylated CDs were applied as chiral selectors in the mobile phase with the achiral column.
- We made ***NMR spectroscopic measurements*** to gain insights in and corroborate findings by chiral HPLC. Spectra were collected on Varian NMR System spectrometers with either 499.9 or 799.7 MHz proton measurement frequencies in mixed solvents corresponding to the HPLC mobile phase at different temperatures.
- We determined the water content of cyclodextrins by ***coulometric Karl Fischer titration*** to correct concentration values for HPLC and NMR measurements.

3. Results

A rapid and high-throughput system has been developed to separate Levonorgestrel API and its impurities at an elevated temperature using a recently introduced, zirconium dioxide-based stationary phase by optimizing the chromatographic conditions (temperature, the identity and portion of organic modifier in the mobile phase, flow rate). My results prove that the selectivity differences due to dissimilar interactions with the methanol and acetonitrile components of the mobile phase persist at elevated temperatures. The theoretical HPLC relationships (van Deemter and van't Hoff equations) are shown to remain safely applicable in the extended temperature range of 50–150 °C. Levonorgestrel and its five impurities can be separated within 2.0–2.5 minutes, so this method may enable in the future the impurity profiling of Levonorgestrel API as well as its selective and rapid determination in drug products.

During the liquid chromatography of Norgestrel enantiomers, α-cyclodextrin applied in the mobile phase did not cause any change in the retention time as compared to the eluent without CD, which fact could be explained by the evaluation of the NMR titration curves of the H4 olefinic, H21 ethinyl and H19 methyl protons. Small value of the stability constant ($K < 5$) indicates only weak interaction which is not sufficient for the recognition and separation of enantiomers in HPLC.

Application of the β-cyclodextrin chiral selector in the HPLC separation gave a more significant decrease in retention time compared to the eluent without CD, but no chiral resolution, this suggests the formation of a weak inclusion complex. ^{1}H NMR titrations showed signal doubling for all the three Norgestrel protons mentioned above, but the

estimated complex stability constants at 25, 40 and 50 °C are again too small ($K < 70$) to induce successful HPLC separation. Stoichiometry of the formed complexes was investigated by using Job's method and we substantiated a previously unidentified 2:1 β-CD/*d*-Norgestrel complex besides the 1:1 one.

Among the native cyclodextrins, γ-CD with a larger cavity size was able to separate Norgestrel enantiomers. Optimization of chromatographic parameters revealed that enantiomeric recognition is favored by applying a lower organic modifier content in the mobile phase, a larger concentration of γ-CD or a lower temperature. Enantiomer-specific stability constants (K_d, K_l) and chiral selectivity (α_c) values were calculated from the results of temperature-dependent HPLC separations and ^{1}H NMR titrations. The results by these independent methods, especially the α_c chiral selectivities showed excellent agreement. We demonstrated that threshold values exist for both the stability constants ($K > 200$) and their ratio ($\alpha_c > 1.1$) to predict successful chromatographic enantioseparation at various temperatures. HPLC data and ^{1}H NMR titrations according to Job's method pointed to the formation of an inclusion complex of 1:1 stoichiometry. 2D NOESY and ROESY spectra provided information on the structure of the *d*-Norgestrel/γ-CD complex.

HPLC investigations with permethylated CDs in the mobile phase lead me to conclude that interactions did exist between Norgestrel and all the three CD derivatives, since significant decreases in retention time were observed. However, chiral recognition of enantiomers did not occur. Non-enantiomeric stability constants were deduced from both HPLC and NMR datasets. The HPLC-based stability constants were by an order of magnitude greater than those calculated from NMR titrations. This indicates that the HPLC-based stability constants may quantify the strong adsorption of the chiral selectors to the stationary phase rather than the formation of the chiral selector/Norgestrel complex. In contrast, the values emerging from NMR evaluations are characteristic solely to the host-guest complex formation in the solution phase and show only weak binding. Preliminary investigations on permethylated CD-containing stationary phases suggested that PM-α-CD (with the smallest cavity size) might give the opportunity to separate Norgestrel enantiomers following a proper method development. HPLC and NMR studies let us to conclude that the elution order is reversed as compared to cases with native CDs, so the pharmaceutically inactive chiral impurity elutes first, which is important in regard to its exact quantitative determination.

4. Theses, novel scientific results

1. I developed for the first time a rapid HPLC method (with an analysis time of 2–2.5 minutes) to separate Levonorgestrel from its five impurities on a modern, zirconium dioxide-based stationary phase designed for elevated temperatures, by optimizing the chromatographic parameters and conditions (temperature, identity and proportion of the organic modifier in the mobile phase, flow rate) [1,3]. With further optimization, this method may enable in the future the impurity profiling of Levonorgestrel API as well as its selective and rapid determination in drug products.
2. My results prove that the selectivity differences due to dissimilar interactions with the methanol and acetonitrile components of the mobile phase persist at elevated temperatures. My studies on Levonorgestrel and its impurities demonstrated for the first time that the theoretical HPLC relationships (specifically, the van Deemter and van't Hoff equations) valid in the usual temperature range can be safely applied in the elevated (50–150 °C) temperature range as well in methanolic mobile phases [1,3]. I have verified for Levonorgestrel and its related impurities that the relationships between structural analogies and the retention parameters remain valid at higher temperatures, and the prediction of retention times is feasible.
3. By ^{1}H NMR technique, binding studies on the H4 olefinic, H21 ethinyl and H19 methyl protons demonstrated for the first time that only weak complexes formed with α- and β-CDs at different temperatures. The obtained data gave explanation why Norgestrel enantiomers could not be separated in HPLC using these CDs [2].
4. Stoichiometric studies according to using Job's method substantiated for the first time the formation of 2:1 β-CD/Norgestrel complex besides the 1:1 one [2].
5. We were first to deduce enantiomer-specific stability constants (K_d, K_l) and chiral selectivity values (α_c) for a range of temperatures from results of HPLC separations and ^{1}H NMR titrations by γ-CD [2]. The results by these independent methods, especially the α_c chiral selectivities showed excellent agreement. We demonstrated that both the K stability constants and their α_c ratios must be above a threshold value ($K > 200$, $\alpha_c > 1.1$) for the successful prediction of chromatographic enantioseparation at various temperatures.

5. List of publications

Full papers emerging from the current PhD work:

[1] Renáta Berta, Mónika Babják, Mária Gazdag: The Role of Temperature in HPLC separations, *Centenary publications by Hungarian Chemical Society Richter factory team*, **2007**, 56-63.

[2] Renáta Berta, Zoltán Szakács, Mónika Babják, Mária Gazdag: The Role of Temperature in Enatioseparation of Norgestrel with Native Cyclodextrins: a Combined HPLC and NMR Study, *Chromatographia*, **2010**, *71*, 35-42.

[3] Renáta Berta, Mónika Babják, Mária Gazdag: A study of some practical aspects of high temperature liquid chromatography in pharmaceutical applications, *Journal of Pharmaceutical and Biomedical Analysis*, **2011**, *54*, 458-462.

Oral presentations:

1. Renáta Berta, Mónika Babják, Mária Gazdag: The Role of Temperature in HPLC separations, *Centenary Conference on Chemistry*, Sopron, 29 May – 1 June 2007.
2. Renáta Berta, Mónika Babják, Mária Gazdag: The Potential of the Chiral Separations with native cyclodextrine by High Temperature Liquid Chromatography Techniques, *Chemistry Lecture Days*, Szeged, 27 – 29 October 2008
3. Renáta Berta, Mónika Babják, Mária Gazdag: Studying the Effect of Elevated Temperature and the Pharmaceutical Analytical Applications in the Fast HPLC Separations, *National Separation Science Meeting 2008*, Sárvár, 5 – 7 November 2008
4. Renáta Berta, Zoltán Szakács: "Colleague-coupled" HPLC – NMR: The beauty and depth of the separation of enantiomers cooperation in the light of separation technique and structure research, *One-day Separation Science Forum 2010*, Budapest, 18 March 2010
5. Renáta Berta: Practical Approach to the Effect of Elevated Temperature on Separation of an Active Pharmaceutical Ingredient, *XXXVI. Pharmaceutical Analysis Postgraduate Colloquium*, Siófok, 8 – 10 April 2010

Posters:

1. Renáta Berta: Study of Chiral Separations by High-Temperature Liquid Chromatography, *Academic Day of Institute of Chemistry ELTE*, Budapest, 9 November 2007

2. Renáta Berta, Mónika Babják, Mária Gazdag: A Study of Chiral Separation Options and Mechanism with Different Cyclodextrins in a chiral API molecule, *National Separation Science Meeting 2008*, Sárvár, 5 – 7 November

3. Renáta Berta, Mónika Babják, Mária Gazdag: Study of the Theoretical and Practical Aspects of the Elevated Temperature for Fast HPLC Separation, *34th International Symposium on High-Performance Liquid Phase Separations and Related Techniques*, Dresda, Germany, 28 June – 2 July 2009

4. Berta R., Szakács Z., Babják M., Gazdag M.: The Role of Temperature in Enantioseparation of Norgestrel with Native Cyclodextrins: a Combined HPLC and NMR Study, *8th Balaton Symposium on High-performance Separation Methods and 15th International Symposium on Separation Science*, Siófok, Hungary, 2 – 4 September 2009

Publications not related to this PhD thesis

Full papers:

1. T. Marek, R. Berta, C. Schür, S. Tautz, P. Kiesel, S. Kunsági-Máté, H.P. Strunk Photolithography and epitaxial lift-off technique: A new preparation method for the transmission electron microscopy in: Physik Mikrostrukturierter Halbleiter, Band 10, 2nd Symposium on Non-Stoichiometric III-V Compounds;
   eds. T. Marek, S. Malzer, P. Kiesel; *Verlag Lehrstuhl für Mikrocharakterisierung*, p. 103-108, **1999**, Erlangen

2. R. Berta: The Water Modeling, *Journal of Teaching in Chemistry* Vol. IX., 20 – 23 September **2003**, Mozaik Publisher

Oral presentations:

1. Renáta Berta: The Role of Stress Stability Testing in the study of active pharmaceutical ingredients, *20th Lectures Day of Young Analysts*, MKE, Budapest, November 2005

2. Renáta G – Berta, Miklós Lőw: With ELTE degree in Richter, *Alchemy Today*, Budapest, 13 December 2007

Poster:

1. Renata Berta: Photolithography and epitaxial lift-off technique in specimen separation for the transmission electron microscopy, *IV. Multinational Congress on Electron Microscopy*, Veszprém, September 5-8, 1999.