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Analysis of Related Substances in Bisoprolol Fumarate on Sub-2-μm Adsorbents

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Summary. The new analytical method of very high pressure liquid chromatography (VHPLC) has been designed for the estimation of related substances in bisoprolol fumarate. Several columns filled with sub-2-μm totally porous and shell adsorbent have been tested and compared during the method development. The columns’ resolution was investigated. The shell adsorbent appears to have smaller resolution than the totally porous adsorbent. The tips and recommendations for the HPLC–VHPLC method transfer are presented. Significant reduction in analysis run time and solvent consumption has been obtained.

Key Words: VHPLC, HPLC, bisoprolol fumarate, resolution factor, shell adsorbent

Introduction

Bisoprolol fumarate is a widely used active substance responsible for systemic action of many cardiac medicine products. This beta-adrenergic antagonist diminishes the effect of catecholamines (i.e., adrenaline) by reducing their concentration and impeding their synthesis. This active substance is commonly used for the treatment of arrhythmia, hypertension, and other cardiovascular diseases. Chemically, it is a derivative of aminopropanol; the full formula of bisoprolol fumarate is presented in Fig. 1. In spite of its popularity, the issue of determining its related substances has not been clearly solved. The latest monograph published in the European Pharmacopeia requires the employment of two separate HPLC gradient elution methods for the estimation of impurities. On the other hand, the commonly used isocratic methods are considered time and solvent consuming.

In recent years, the new technique of very high pressure liquid chromatography (VHPLC) has been widely studied. VHPLC enables considerable reduction of the time of analysis. In this technique, the column is packed with sub-2-μm particles that are totally or superficially porous. The reduction of the size of the adsorbent causes the decrease of mass transfer resis-
tances and increases column efficiency. Further reduction in mass transfer resistances and increase in column efficiency are possible by introducing superficially porous particles [1–8]. The increase in column efficiency enables applying higher flow rates of the mobile phase, significantly reducing the analysis time. The effectiveness of the shell particles has been examined in isocratic as well as in gradient elution conditions [9, 10]. In both cases, column efficiency increases considerably over traditional HPLC, especially for shell particles, which means that the height equivalent to theoretical plate (HETP) is smaller than in classical HPLC and the analysis time can be much shorter.

Fig. 1. Structures of bisoprolol fumarate and its related substances
It should be noted that the application of very small particles requires very high inlet pressure to pump the mobile phase through the bed. This can cause the development of axial and, what is especially unfavorable, radial temperature gradient [11–13].

The radial temperature gradient and a serious decrease of column efficiency can be minimized when the column works under conditions similar to adiabatic.

The aim of this study was to develop a fast VHPLC analytical method appropriate for the analysis of all potential impurities of bisoprolol. For this purpose, several types of chromatographic columns with sub-2-μm particles that are totally or superficially porous were tested. The second aim was to explain theoretically the observed poorer resolution of columns filled with the shell adsorbent in comparison with totally porous adsorbents.

**Theory**

The column efficiency is traditionally compared by presenting the number of theoretical plates or HETP. The number of theoretical plates $N$ can be calculated from the relation [5, 14]

$$N = \frac{L}{\text{HETP}} = \frac{\mu_1^2}{\mu_2}$$

(1)

where $\mu_1$ is the first absolute moment and $\mu_2$ is the second central moment of the chromatographic peak.

Assuming that the adsorption process is instantaneous, which is generally accepted, the first absolute and the second central moments for the totally porous adsorbent as well as for an adsorbent with an inert core can be calculated from the following equations [5]:

$$\mu_1 = (1 + k_1) \frac{L \varepsilon_e}{u}$$

(2)

$$\mu_2 = \frac{L \varepsilon_e}{u} \left( \frac{2D_k \varepsilon_e (1 + k_1)^2 + 2(k_1)^2}{3} \frac{F'}{R_e} L \varepsilon_e \frac{1}{k_{\text{ext}}} + \frac{R_e}{5D_{\text{eff}}} \frac{1 + 2 \rho + 3 \rho^2 - \rho^3 - 5 \rho^4}{(1 + \rho + \rho^2)^2} \right)$$

(3)

where

$$k_1 = F' \left( \varepsilon_p + (1 - \varepsilon_p) H \right) \left( 1 - \rho^3 \right); \quad F' = \frac{1 - \varepsilon_e}{\varepsilon_e}; \quad k_p = \frac{1 - \varepsilon_p}{\varepsilon_p} H$$

(4)

The meanings of the symbols are given in the section Notation.
The first moment is equal to the retention time (RT) of the chromatographic peak.

The second central moment is a measure of the peak dispersion due to axial dispersion (the term with $D_L$ in eq. (3)), external mass transfer resistances (the term with $1/k_{ext}$), and internal mass transfer resistances characterized by last term in eq. (3).

From eqs (1) and (3), it follows that the HETP for the instantaneous adsorption process always decreases when the ratio $\rho$ of the radius of the inner solid core $R_i$ to the radius of the particle $R_e$ is increasing [5] – it means that the column becomes more effective when the shell thickness decreases.

However, the most important measure of a chromatographic separation power is the resolution $R_s$ between the different compounds.

The resolution $R_s$ of two compounds can be calculated from the first and second moments of the chromatographic peaks as [9]

$$R_s = \frac{|\mu_{1a} - \mu_{1b}|}{2\sqrt{\mu_{2a} + \mu_{2b}}}$$

(5)

where $a$ and $b$ denote the component a or b.

Depending on the values of the physicochemical parameters, the maximum resolution can be obtained for the dimensionless solid core radius $\rho$ in the interval [0–1] – see Refs [8] and [15], despite the fact that maximum efficiency is reached for $\rho$ increasing to 1. Equations (1) and (5) coupled with eqs (2) and (3) were applied for the theoretical analysis of the efficiency and resolution of the columns used in this work.

**Experimental**

**Instrumentation**

Two different LC systems were used during the study: One was a Waters 2695 (Waters Corporation, Milford, MA) separation module (max. operating pressure 345 bar) with a photodiode array detector (PDA 2998); and the other was a Dionex Ultimate 3000 RS (Dionex Corporation, Sunnyvale, CA) (maximum operating pressure 800 bar) with a diode array detector (DAD-3000RS).

Columns: In the experiment, different C-18 columns were used. EC Nucleosil columns were supplied by Macherey-Nagel; XTerra and BEH columns were supplied by Waters; Dionex Acclaim RSLC columns were obtained from Dionex Corporation; Kinetex core–shell columns were supplied
by Phenomenex; and Chromolith Performance columns were supplied by Merck KGaA, Germany.

Materials

Methanol and acetonitrile, both of HPLC grade, were obtained from Merck GmbH and LabScan. Triethylamine (≥99.5%, HPLC grade) was supplied by Sigma Aldrich. Orthophosphoric acid (85%) and anhydrous potassium dihydrogen phosphate were supplied by POCH, Poland.

Bisoprolol fumarate working standard and its impurities (A, L, B, X) were obtained from ICN Polfa Rzeszów S.A. Impurity A (“benzalcohol”) is a degradation product of the active substance arising from the hydrolysis of the benzyl ether linkage; further oxidation of this impurity leads to the formation of impurity L (“benzaldehyde”). Impurity B (“n-propoxy”) is a process impurity produced during the synthesis, where isopropanol is used as starting material and the solvent may contain small quantities of propoxyethanol. Impurity X is also a degradation product, arising from the formylation of bisoprolol fumarate.

The chemical names of impurities are listed below; their structures are presented in Fig. 1.

- **Impurity A**: (RS)-1-(4-hydroxymethyl-phenoxy)-3-isopropylamino-propan-2-ol
- **Impurity B**: (RS)-1-isopropylamino-3-[4-(2-propoxy-ethoxymethyl)phenoxy]propan-2-ol
- **Impurity L**: 4-[[2(RS)-2-hydroxy-3-(isopropylamino) propyl]oxy] benzaldehyde
- **Impurity X**: N-(2-hydroxy-3-(4-(2-isopropoxyethoxymethyl)phenoxy) propyl)-N-isopropyl formamide

System suitability reference solutions A and B and impurity G of bisoprolol were supplied by LGC Standards.

Impurity G is (2RS)-1-[4-[[2-(isopropoxyethoxy)methoxy]methyl] phenoxy]-3-isopropylamino propan-2-ol.

Impurity G is also a process impurity arising during the synthesis of bisoprolol base.

Water for analysis was purified with an UltraPure Water Systems (Millipore, Billerica, MA).

The names and structures of potential impurities correspond to those described in the European Pharmacopeia 6.4 Bisoprolol Fumarate Monograph (04/2008:1710), except impurity X which was identified and synthesized at ICN Polfa Rzeszów S.A.
Chromatographic Conditions

The chromatographic conditions are specified in *Tables I* and *II*.

*Table I. Chromatographic conditions for HPLC methods*

<table>
<thead>
<tr>
<th>Method</th>
<th>Column</th>
<th>Mobile phase A and B:</th>
<th>Gradient elution program</th>
<th>Flow rate (mL min⁻¹)</th>
<th>Wavelength (nm)</th>
<th>Column temperature (°C)</th>
<th>Sample volume (μL)</th>
<th>Run time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method A from European Pharmacopeia</td>
<td>Waters, X-Terra C18, 5 μm, 4.6 × 250 mm</td>
<td>Acetonitrile + buffer of pH 4.2 (10 + 90 v/v)</td>
<td>Time (min)</td>
<td>95–10</td>
<td>1.0</td>
<td>UV 225</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Method B from European Pharmacopeia</td>
<td>Macherey-Nagel, EC Nucleosil 100-5 C18 HD, 5 μm, 4.6 × 250 mm</td>
<td>Acetonitrile + buffer of pH 4.2 (75 + 25 v/v)</td>
<td>Time (min)</td>
<td>90–20</td>
<td>0.8</td>
<td>UV 225</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>In-house method</td>
<td>Macherey-Nagel, EC Nucleosil 100-5 C18 HD, 5 μm, 4.6 × 250 mm</td>
<td>Phosphate buffer solution of pH 5.5</td>
<td>Time, (min)</td>
<td>57</td>
<td>1.0</td>
<td>UV 225</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Method D from Pharmeuropa</td>
<td>Chromolith performance RP-18e 5 μm, 100 × 4.6 mm Columns 1 and 2 are connected in series</td>
<td>10 g L⁻¹ solution of phosphoric acid in acetonitrile</td>
<td>Time (min)</td>
<td>95</td>
<td>UV 225</td>
<td>UV 225</td>
<td>UV 225</td>
<td>UV 225</td>
</tr>
</tbody>
</table>
Table II. VHPLC method conditions

<table>
<thead>
<tr>
<th>Columns type</th>
<th>Time (min)</th>
<th>Mobile phase A, % (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient program for 50- and 100-mm columns</td>
<td>0–0.1</td>
<td>25–40</td>
</tr>
<tr>
<td></td>
<td>0.1–3.4</td>
<td>40–45</td>
</tr>
<tr>
<td></td>
<td>3.4–5.3</td>
<td>45–90</td>
</tr>
<tr>
<td></td>
<td>5.3–5.4</td>
<td>90–25</td>
</tr>
<tr>
<td></td>
<td>5.4–8</td>
<td>25</td>
</tr>
<tr>
<td>Gradient program for 150-mm columns</td>
<td>0–0.1</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.1–0.2</td>
<td>25–40</td>
</tr>
<tr>
<td></td>
<td>0.2–3.4</td>
<td>40–45</td>
</tr>
<tr>
<td></td>
<td>3.4–7.3</td>
<td>45–90</td>
</tr>
<tr>
<td></td>
<td>7.3–8.4</td>
<td>90–25</td>
</tr>
<tr>
<td></td>
<td>8.4–13</td>
<td>25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>UV 225</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Column temperature (°C)</th>
<th>50</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Run time</th>
<th>100- and 50-mm columns – 8 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>150-mm columns – 13 min</td>
</tr>
</tbody>
</table>

Test and Reference Solutions

Test solutions

*Bisoprolol fumarate solution.* Bisoprolol fumarate (24.5 mg) was dissolved in the mobile phase (acetonitrile + buffer of pH 4.2 (10:90, v/v)) and diluted to 25.0 mL in the same solvent.

*Bisoprolol fumarate spiked solution.* Bisoprolol fumarate (25.2 mg) was dissolved in the mobile phase (acetonitrile + buffer of pH 4.2 (10: 90, v/v)); this mixture was spiked with impurities A, L, G, B and X at the 0.5% level and diluted to 25.0 mL with the same mobile phase.

Reference solutions

*Reference solution for method A.* The content of a vial of bisoprolol for system suitability method A, CRS (abbreviations are explained in the section Notation) was dissolved in 1.0 mL of mobile phase A (acetonitrile + buffer of pH 4.2 (10: 90, v/v)).

*Reference solution for method B.* Content of a vial of bisoprolol for system suitability method B CRS was dissolved in 1.0 mL of the solvent mixture (acetonitrile and water 20: 80, v/v).
Results and Discussion

Migration from HPLC to VHPLC

Analytical Methods (A and B) from the European Pharmacopeia

According to the latest issue of the European Pharmacopeia, complete analysis of related substances in bisoprolol fumarate requires the employment of two separate gradient-elution HPLC methods. Method A has been designed for the analysis of six impurities, namely, A, B, C, D, E, and F; however, the monograph recommends disregarding the peak due to impurity G. Method B is a completion of method A, and allows the estimation of the content of 11 impurities (out of 15); according to the monograph, this analysis is not suitable for the estimation of impurity E. Application of two methods for the routine analysis of frequently used active substance is not very convenient. A single run of method A takes at least 60 min and requires over 23 mL of acetonitrile, and a single run of method B takes 50 min and requires over 15 mL of that organic solvent. In summary, the analysis time for both methods is 110 min and needs at least 38 mL of acetonitrile; additionally, they are time and solvent consuming. Another difficulty when using methods from the European Pharmacopeia is that they use a low concentration of organic solvent in the initial stage of the analysis, and then strong gradient elution at the end, which are favorable conditions for generating ghost peaks [16]. Organic impurities contaminating, for example, purified water may be strongly retained in the stationary phase at the initial stage of the analysis; the following increase of concentration of the organic solvent elutes those impurities, giving false signals referred to in the literature as ghost peaks. In fact, at the end of the strong gradient stage, both methods give rise to a significant number of false signals which may finally affect the estimation of unknown impurities.

Method C

Before the first monograph on bisoprolol fumarate was published, a method C had been applied for the analysis of impurities in the active substance. It is suitable for the estimation of all major impurities. Moreover, good resolution allowed the observation of two peaks due to impurity E (an alkene), which were identified as the cis and trans isomers. An analysis time of 30 min was determined by the elution time of the last organic impurity (impurity X), and single run required over 14 mL of methanol.
Method D from Pharmeuropa

The issue of Pharmeuropa published in January 2010 consists of a new monograph for bisoprolol fumarate, where a single HPLC analytical method, selective to all potential impurities, replaces methods A and B. The new method D requires two separate columns filled with monolithic octadecylsilyl silica gel, which are then combined in series. Good resolution between peaks due to bisoprolol and impurity G (the closest eluting substances) has been achieved. The overall analysis time, including re-equilibration to initial conditions, was approximately 45 min and required 14.5 mL of acetonitrile.

In summary, the RTs of the active pharmaceutical ingredient (API, bisoprolol) are between 13 and 18 min in the above-mentioned methods. The relative retention time (RRt) of the closest impurity G was between 1.04 and 1.2. However, the best resolution between impurity G and the API has been obtained for method D – 3.05, whereas other methods were not able to fully separate those compounds and only the \( H_p/H_v \) ratio could be calculated.

VHPLC Method Transfer and Development

VHPLC offers the advantage over HPLC by reducing both solvent consumption and analysis time without loss of resolution. In this work, we have focused on the development of a new analytical method suitable for the analysis of all related substances in bisoprolol fumarate.

The VHPLC method conditions are presented in Table II, and the list of columns tested is given in Table III.

The VHPLC columns are packed usually with sub-2-\( \mu \)m silica particles, which substantially increases the operating backpressure; however, this particle size offers many advantages. For example, it ensures minor changes in HETP even when the linear velocity of the mobile phase changes significantly; this is how one can reduce the analysis time without the loss of the separation capabilities. The optimal linear velocity range for sub-2-\( \mu \)m particles ranges between 3 and 7 mm s\(^{-1} \) [17]. In our experiment, the volume flow rate was 0.5 mL min\(^{-1} \), which guarantees the linear velocity to be in the above-mentioned range. The exceptions were the 150-mm columns (see the next section). The injection volume should be adapted taking into consideration VHPLC column volume; this operation prevents the column from getting overloaded. The volume is scaled down according to eq. (6).

\[
V_{\text{VHPLC,injection}} = \frac{V_{\text{original,injection}} \times V_{\text{VHPLC,original}}}{V_{\text{original,original}}}
\]  

(6)
Table III. List of columns tested during method development

<table>
<thead>
<tr>
<th>Column type</th>
<th>Dimensions</th>
<th>Particle size (μm)</th>
<th>Injection volume (μL)</th>
<th>Flow rate (mL min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetex, C18, core-shell particle, Phenomenex, inert core radius, ( R_i = 0.6 ) μm</td>
<td>150 mm × 2.1 mm</td>
<td>1.7</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>100 mm × 2.1 mm</td>
<td>1.7</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>50 mm × 2.1 mm</td>
<td>1.7</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Acquity UPLC, BEH C18, Waters</td>
<td>150 mm × 2.1 mm</td>
<td>1.7</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>100 mm × 2.1 mm</td>
<td>1.7</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>50 mm × 2.1 mm</td>
<td>1.7</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Acclaim® (RSLC), C18, Dionex</td>
<td>150 mm × 2.1 mm</td>
<td>2.2</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>100 mm × 2.1 mm</td>
<td>2.2</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>50 mm × 2.1 mm</td>
<td>2.2</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The obtained chromatograms for column lengths of 100 and 150 mm are presented in Figs 2 and 3, respectively. The solid line represents the concentration profiles, and the dashed line is the gradient program. The gradient program has been designed to allow analysis of substances that significantly differ in polarity. Initial conditions with low concentration of the organic solvent enable good resolution between polar impurities A and L and fumaric acid, and further increment of methanol concentration shortens the RT of bisoprolol as well as impurities B and G. The most strongly retained impurity X may be quickly eluted by increasing the concentration of methanol to 90%; the last stage of the gradient program is restoring the initial conditions and a short equilibration time before another injection.

Comparing impurities G and B and bisoprolol, we can clearly notice the structural similarity between them. Structurally related substances are often characterized by similar physical and chemical properties, which make them difficult to analyze by liquid chromatography. Thus the crucial effort was to fully separate those three compounds, which determined the final success of the method transfer. Comparison of asymmetry factors and mobile phase consumption for all tested columns is presented in Table IV.
Fig. 2. Results obtained with 100-mm columns

a) column BEH C18 100 mm, Waters
b) column Acclaim C18 100 mm, Dionex
c) column Kinetex C18 100 mm, Phenomenex
Fig. 3. Results obtained with 150-mm columns

a) column BEH C18 150 mm, Waters
b) column Acclaim C18 150 mm, Dionex
c) column Kinetex C18 150 mm, Phenomenex
Table IV. Analysis times and solvent consumption for VHPLC methods

<table>
<thead>
<tr>
<th>Column type</th>
<th>Column dimensions</th>
<th>Single run time (min)</th>
<th>Asymmetry (EP) of the main peak</th>
<th>Mobil phase consumption (mL)</th>
<th>Organic solvent consumption (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kinetex, C18, core–shell particle, Phenomenex</strong></td>
<td>150 mm × 2.1 mm; 1.7 μm</td>
<td>13</td>
<td>2.14</td>
<td>3.9</td>
<td>1.84</td>
</tr>
<tr>
<td></td>
<td>100 mm × 2.1 mm; 1.7 μm</td>
<td>8</td>
<td>1.55</td>
<td>4.0</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>50 mm × 2.1 mm; 1.7 μm</td>
<td>8</td>
<td>1.51</td>
<td>4.0</td>
<td>1.71</td>
</tr>
<tr>
<td><strong>Acquity UPLC, BEH C18, Waters</strong></td>
<td>150 mm × 2.1 mm; 1.7 μm</td>
<td>13</td>
<td>1.94</td>
<td>3.9</td>
<td>1.84</td>
</tr>
<tr>
<td></td>
<td>100 mm × 2.1 mm; 1.7 μm</td>
<td>8</td>
<td>1.47</td>
<td>4.0</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>50 mm × 2.1 mm; 1.7 μm</td>
<td>8</td>
<td>1.26</td>
<td>4.0</td>
<td>1.71</td>
</tr>
<tr>
<td><strong>Acclaim (RSLC), C18, Dionex</strong></td>
<td>150 mm × 2.1 mm; 2.2 μm</td>
<td>13</td>
<td>1.96</td>
<td>3.9</td>
<td>1.84</td>
</tr>
<tr>
<td></td>
<td>100 mm × 2.1 mm; 2.2 μm</td>
<td>8</td>
<td>1.52</td>
<td>4.0</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>50 mm × 2.1 mm; 2.2 μm</td>
<td>8</td>
<td>1.51</td>
<td>4.0</td>
<td>1.71</td>
</tr>
<tr>
<td><strong>Method A, European Pharmacopeia</strong></td>
<td>250 × 4.6 mm; 5 μm</td>
<td>60</td>
<td>–</td>
<td>60.0</td>
<td>&gt;23</td>
</tr>
<tr>
<td><strong>Method B, European Pharmacopeia</strong></td>
<td>250 × 4.6 mm; 5 μm</td>
<td>50</td>
<td>–</td>
<td>50.0</td>
<td>&gt;15</td>
</tr>
<tr>
<td><strong>Method C</strong></td>
<td>250 × 4.6 mm; 5 μm</td>
<td>30</td>
<td>–</td>
<td>24.0</td>
<td>&gt;14</td>
</tr>
<tr>
<td><strong>Method D, Pharmeuropa</strong></td>
<td>250 × 4.6 mm; 5 μm</td>
<td>45</td>
<td>–</td>
<td>45.0</td>
<td>&gt;14</td>
</tr>
</tbody>
</table>

150-mm Columns

The Acquity BEH column 150 mm × 2.1 mm has been tested with a flow rate 0.3 mL min⁻¹, generating 595 bar total (in column and tubing) backpressure in the initial stage of analysis. During the gradient program, the maximum backpressure moved closer to 800 bar, which was the highest operating pressure for our VHPLC system; that is why higher flow rates could not be applied. The injection volume was set to 1.4 μL for all 150-mm columns. The fastest eluting compound was fumaric acid, followed by impurity A and impurity L, all baseline separated. However, probably due to the strong re-

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tention mechanism, substantial peak tailing of bisoprolol (RT = 6.09 min) occurred, and the calculated asymmetry factor (according to Eur. Ph.) was 1.94. Additionally, bisoprolol eluted at the strong gradient stage, which made the integration of impurities G and B even more difficult, with RRt, 1.08 and 1.11, respectively, eluting on the main peak’s tail. These results were compared with results obtained for the Kinetex 150 mm × 2.1 mm column. Kinetex core–shell particles are not fully porous; they consist of a solid nonporous core covered by a porous shell. Such a design is meant to reduce the diffusion time of analytes inside the pores, which enables faster mass transfer and reduced band broadening. The results obtained for the Kinetex 150 mm column confirm the faster mass transfer. However, the shorter RT (for bisoprolol, RT = 5.18 min on Kinetex and RT = 6.09 min for the BEH column) points to a smaller column total adsorption capacity when compared with the Acquity BEH column. The asymmetry (Eur. Ph.) of the bisoprolol peak was 2.14. Visually, impurities G, B, and bisoprolol were not baseline separated.

Another tested column was Dionex Acclaim (RSLC) 150 mm; the smallest particle size available for this type of column is 2.2 μm. Bisoprolol eluted in 6.80 min. On the chromatogram of spiked solutions, one could observe good resolution between fumaric acid and impurities A and L; however, impurities B, G, and bisoprolol were not fully separated. Again, bisoprolol was retained more strongly and eluted in the strong gradient stage; these results are similar to those obtained with the Acquity BEH 150-mm column. In all attempts, the strongest retained analyte was impurity X, which could elute from the column only after a significant increase in the concentration of the organic solvent. All 150-mm columns were tested under the same chromatographic conditions: the run time was set up to 13 min, which included re-equilibration stage at the end of analysis, and total mobile phase consumption was 3.9 mL (only 1.84 mL of methanol). Taking into consideration the maximum operating pressure for the Dionex UPLC system (800 bar), we could double the flow rate and compare the results. Analysis with a flow 0.6 mL min⁻¹ gave good separation between fumaric acid and impurities A and L. Impurities G and B are clearly separated, but both elute at the main peak’s tail. The last eluting impurity was impurity X, at 7.02 min.

100-mm Columns

Acquity BEH 100 mm × 2.1 mm, 1.7 μm particle size, was the next tested column. The analysis time could be reduced additionally from 13 to 8 min compared to 150-mm columns; smaller column dimensions affect also the injection volume, which was set to 1.0 μL. The elution order remained un-
changed, namely, fumaric acid, impurity A, and impurity L, with good separation. A smaller injection volume and a shorter column size result in a smaller peak asymmetry (1.47) when compared to the 150-mm BEH column. On the chromatogram of the spiked solution, almost baseline separation could be observed between the peaks due to bisoprolol (2.98 min) and impurity G. Impurity X is the last compound eluting from the column, with an RT of 5.92 min. The same chromatographic conditions were applied for the Kinetex 100-mm column; this time, however, we observed worse resolution for fumaric acid and impurity A, which were not fully separated. Impurities B and G gave a single peak with two apexes rather, although both were separated from the main peak. The asymmetry value was 1.55 for the bisoprolol peak, which was worse than for the BEH 100-mm column. Mass transfer was still faster, but it does not compensate for the lack of resolution. The last 100-mm column tested was the Acclaim RSLC. Surprisingly, in spite of the larger particle size (2.2 \(\mu\)m), we observed baseline separation for fumaric acid and impurities A and L; it was a much better performance than for the smaller 1.7-\(\mu\)m core–shell particles. The peak due to bisoprolol had a higher tailing factor, which might affect the resolution between the main peak and the peak due to impurities G and B. All 100-mm columns were tested under the same chromatographic conditions: the analysis time was 8 min, and total mobile phase consumption was 4.0 mL (only 1.71 mL of methanol per run). In fact, the analytical method with the BEH 100-mm column could be used in routine analysis of bisoprolol fumarate, reducing time and the organic solvent consumption significantly.

50-mm Columns

When using 50-mm columns, the injection volume was scaled down to 0.5 \(\mu\)L, which is the smallest recommended injection volume for VHPLC systems. The gradient program and analysis time were left unchanged as for the 100-mm columns. Under these conditions, all tested 50-mm columns presented much poorer separations (figures not presented). That is why 100-mm columns are recommended for better resolution and 50-mm columns for faster coarse analysis. With the Acquity BEH column, we could not separate fumaric acid and impurities A and L; also, impurities B and G were eluted as single peaks, which are in turn partially coeluted with the main peak. Finally, impurity X eluted last, as expected, with an RT of 4.69 min. Kinetex core–shell particle column gave similar results. Lack of resolution between fumaric acid and impurity A and L, as well as coelution of impurities G and B, make this column useless for routine analysis. Again, the Dionex column gave better results than Kinetex. Fumaric acid only
slightly coeluted with impurity A, whereas impurity L was baseline-separated; however, we observed a lack of resolution between bisoprolol, impurity G, and impurity B. All 50-mm columns were tested under the same chromatographic conditions: the analysis time was 8 min, and the total mobile phase consumption was 4.0 mL, including 1.71 mL of methanol.

**Column Resolutions**

From an analysis of the retention on 100-mm columns (Fig. 2) of impurities B and G, it is clearly visible that the poorest resolution was obtained for the Kinetex core–shell column, which is expected to be the most efficient. For comparison purposes, we repeated the separation on the same set of columns but under isocratic conditions – see Fig. 4. The methanol concentration was 37% (v/v), which could ensure visible resolution between API and the impurities G and B. Lower quantities of methanol gave no significant improvement in resolution, but required much longer analysis times, as the very nonpolar impurity X was strongly retained on the column. Also, for isocratic elution the resolution was poorest for the Kinetex core–shell column.

The poorer resolution of the Kinetex column in comparison with Acquity BEH and Acclaim RSLC can be explained by analyzing Eq. (5). As can be seen, the resolution is proportional to the difference of the first moments (difference of RTs) and to column efficiency (the smaller the second moment, the higher the column efficiency). The Kinetex column offers the highest internal mass transfer (lowest mass transfer resistances) due to the narrow shell; however, it is accompanied by a reduction in total column sorption capacity and shorter RTs. Under specific situations, the reduction of RT dominates over the increase in column efficiency, and finally resolution is poorer for the shell column.

For qualitative comparison of the efficiency and separation power $R_s$ of the discussed chromatographic systems, eqs (1) and (5) coupled with eqs (3) and (4) were used. In this comparison, which was done for impurities B and G, we neglected the heat effect and peak broadening in the connecting tubes. The parameters needed to calculate $N$ and $R_s$ were evaluated using commonly accepted methods [14]. The Henry constant was obtained from eq. (2); the mass transfer coefficient $k_{ext}$ from Wilson and Geankoplis correlation [18]; the molecular diffusivity $D_m$ from [19], and the dispersion coefficient from the Wen and Fan model [20].
Fig. 4. Results obtained with 100-mm columns (isocratic method)
The effective diffusion coefficient was calculated from the classical equation

\[ D_{\text{eff}} = \frac{D_e \varepsilon_p}{\tau} \]  

where \( \tau \) is the tortuosity factor estimated from the following relationship [14]:

\[ \tau = \frac{(2 - \varepsilon_p)^2}{\varepsilon_p} \]  

and particle porosity was obtained from

\[ \varepsilon_p = \frac{\varepsilon_{\tau} - \varepsilon_e}{1 - \varepsilon_e} \]  

The total porosity was calculated from the version of eq. (2) for inert species. In this case, fumaric acid can be regarded as the inert component. The retentions of fumaric acid and the impurities B and G were corrected by subtracting the extra volume dead time.

The unknown value, that is, external porosity, was calculated from the Blake, Kozeny, and Carman equation [21]

\[ \frac{\Delta p}{L} = \xi \mu \eta \frac{(1 - \varepsilon_e)^2}{\varepsilon_e^2 d_p^2} \]  

by measuring the drop on the column \( \Delta p \) and assuming \( \xi = 150 \) [21].

The values of the calculated parameters needed for estimation of \( N \) and \( R_s \) are summarized in Table V.

**Table V.** Parameter values for calculation resolution factor and values of \( N \) and \( R_s \)

<table>
<thead>
<tr>
<th>Column</th>
<th>Kinetex</th>
<th>Acquity BEH</th>
<th>Acclaim RSLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \varepsilon_t )</td>
<td>0.503</td>
<td>0.589</td>
<td>0.618</td>
</tr>
<tr>
<td>( \varepsilon_e )</td>
<td>0.385</td>
<td>0.390</td>
<td>0.356</td>
</tr>
<tr>
<td>( \varepsilon_p )</td>
<td>0.295</td>
<td>0.327</td>
<td>0.407</td>
</tr>
<tr>
<td>Henry constant for B</td>
<td>8.32</td>
<td>9.07</td>
<td>11.9</td>
</tr>
<tr>
<td>Henry constant for G</td>
<td>8.79</td>
<td>9.77</td>
<td>12.7</td>
</tr>
<tr>
<td>( D_L ) (m² s⁻¹) for B</td>
<td>4.00 × 10⁻⁹</td>
<td>3.95 × 10⁻⁹</td>
<td>5.73 × 10⁻⁹</td>
</tr>
<tr>
<td>( D_L ) (m² s⁻¹) for G</td>
<td>4.04 × 10⁻⁹</td>
<td>3.99 × 10⁻⁹</td>
<td>5.92 × 10⁻⁹</td>
</tr>
<tr>
<td>( D_m ) (m² s⁻¹) for B</td>
<td>5.10 × 10⁻¹⁰</td>
<td>5.10 × 10⁻¹⁰</td>
<td>5.10 × 10⁻¹⁰</td>
</tr>
<tr>
<td>( D_m ) (m² s⁻¹) for G</td>
<td>4.87 × 10⁻¹⁰</td>
<td>4.87 × 10⁻¹⁰</td>
<td>4.87 × 10⁻¹⁰</td>
</tr>
<tr>
<td>( k_{\text{ext}} ) (m² s⁻¹) for B</td>
<td>0.182</td>
<td>0.180</td>
<td>0.228</td>
</tr>
<tr>
<td>( k_{\text{ext}} ) (m² s⁻¹) for G</td>
<td>0.188</td>
<td>0.186</td>
<td>0.241</td>
</tr>
<tr>
<td>( N )</td>
<td>3800</td>
<td>5700</td>
<td>5500</td>
</tr>
<tr>
<td>( R_s )</td>
<td>0.7</td>
<td>1.2</td>
<td>1.1</td>
</tr>
</tbody>
</table>
In Table V, the calculated values of the number of theoretical plates and resolution factors for all columns are also given. As can be seen, surprisingly, the number of theoretical plates is the smallest for the Kinetex column. It should be noted that values of the kinetic parameters for the Kinetex and Acquity BEH columns are almost the same, which means that their dispersion and external mass transfer resistances are very similar. The internal mass transfer resistances have to be the smallest for Kinetex because of its narrow shell. So, for the smallest Kinetex column, efficiency is responsible for the smallest value of Henry constant obtained for this column.

The theoretical model for the calculation of the resolution factor $R_s$ (eq. (5) coupled with expression for the first absolute moment and second central moment) correctly forecasts the resolution of the three analyzed columns. However, the obtained $R_s$ values overestimated the real column resolution (which is easily seen in Fig. 4). Despite this, it seems that eq. (5), together with expressions (2) and (3), can be used for the estimation of the resolution power of the columns under interest and primary selection of these columns.

**Conclusions**

In spite of the popularity of bisoprolol fumarate, all applied methods for the analysis of its impurities take long and are inconvenient, even those published in the European Pharmacopeia. Similar physical and chemical properties of the related substances make it even more difficult to determine. The new VHPLC with the sub-2-μm particle size stationary phase enables fast and efficient analysis, significantly reducing run times and solvent consumption, lowering solvent and waste-disposal costs.

Different types of columns were compared during method development, and the best separation was obtained for fully porous silica particles of 1.7 μm. Core–shell particles in fact increase mass transfer, but show worse separations when compared to other tested stationary phases. The 2.2-μm column package may result in slightly stronger peak tailing, but higher flow rates may be applied.

The proposed theoretical model enables the qualitative comparison of the resolution power for the columns filled with totally porous as well as shell adsorbent.

**Notation**

API – Active pharmaceutical ingredient  
CRS – Certified reference standard  
$d_p$ – Particle diameter
$D_{\text{eff}}$ – Effective particle diffusivity  
$D_m$ – Molecular diffusivity  
$D_L$ – Axial dispersion coefficients  
Eur. Ph. – European Pharmacopeia  
$F'$ – Phase ratio  
$H$ – Henry constant  
$H_P$ – Height above the baseline of the peak due to impurity G  
$H_v$ – Height above the baseline of the lowest point of the curve separating peak G from the main peak  
$H_P/H_v$ – Peak to valley ratio  
$k_{\text{ext}}$ – External mass transfer coefficient  
$L$ – Column length  
$N$ – Number of theoretical plates  
$R_s$ – Resolution  
$R_i$ – Radius of inert solid core  
$R_e$ – Particle radius  
$RT$ – Retention time  
$RRt$ – Relative retention time  
$S_f$ – Symmetry factor  
$t$ – Time  
$u$ – Superficial velocity

**Greek Symbols**

$\varepsilon_e$ – External porosity  
$\varepsilon_t$ – Total column porosity  
$\varepsilon_p$ – Particle porosity  
$\mu_1$ – First absolute moment  
$\mu_2'$ – Second central moment  
$\eta$ – Viscosity  
$\rho$ – Ratio of the radius of the inner solid core, $R_i$, to the radius of the particle, $R_e$  
$\tau$ – Tortuosity

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References


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