Liquid Chromatographic Separation and Thermodynamic Investigation of Mirabegron Enantiomers on a Chiralpak AY-H Column

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Liquid chromatographic separation of mirabegron enantiomers on Chiralpak AY-H, a column coated with amylose tris-(5-chloro-2-methylphenylcarbamate) as a chiral stationary phase, was studied under normal phase conditions. The influence of ethanol content (30–45%) and column temperature (20–40°C) on retention, resolution and separation were evaluated. Apparent thermodynamic parameters deduced from Van’t Hoff plots were used to understand chiral separation mechanisms, and the chiral separation was enthalpy driven. The optimized chromatographic conditions were using a mixture solution of n-hexane, ethanol and diethyl amino (55 : 45 : 0.1, v/v/v) as a mobile phase at a flow rate of 1.0 mL/min. The column temperature and UV detector were set at 35°C and 254 nm, respectively. The method was validated to be simple, accuracy, sensitive and robust according to the ICH guidelines, and it was suitable for the routine quality control of mirabegron enantiomers for pharmaceutical industries.

Introduction

Mirabegron (CAS 223673-61-8), designated as 2-amino-N-[4-[2-[[2R]-2-hydroxy-2-phenyl-ethyl]amino]-ethyl]phenyl]-4-thiazoleacetamide, is an orally β3-adrenoceptor (AD) agonist for the treatment of overactive bladder symptoms (1, 2). By activation of β3-AD, mirabegron (MI-I) facilitates bladder filling and reduces micturition frequency with better safety therapy than other antimuscarinic drugs (3, 4), and it is proved to be effective and well tolerated with once-daily doses of 50 or 100 mg (5).

During the synthesis of MI-I, the (S)-enantiomer (MI-II, Figure 1) is obtained as chiral impurity affecting the drug quality. Owing to the differences between chiral substances in receptor–ligand interactions and functional biological effects, the enantiomers often exhibit various pharmacological, pharmacokinetic and even toxicological properties (6, 7). Determination and quantity analysis of enantiomeric composition are key points for chiral drugs in the pharmaceutical process (8, 9).

A literature survey reveals that only several literatures reported the analytical methods for the determination of MI-I and its metabolites (10–12), and none of the chiral separation have been available for MI enantiomers till now. Therefore, it is necessary to develop a simple, rapid and sensitive enantioselective HPLC method for routine quality control of MI-I during drug development and storage.

The present study focuses on the separation and quantification analysis of MI-I and its enantiomer based on the chiral stationary phases (CSPs). The influence on chromatographic retention, separation and resolution were investigated by using different chiral columns (Chiralpak AD-H and AY-H), polar modifiers, additives and column temperatures. The established normal phase HPLC was then validated with respect to accuracy, precision, limits of detection (LOD) and quantification (LOQ), linearity and robustness in accordance to the ICH guidelines. Furthermore, the apparent thermodynamic parameters were derived from Van’t Hoff plots to understand the possible separation mechanisms.

Experimental

Chemicals and reagents

Mirabegron and its (S)-enantiomer, 99.8% purity by HPLC, were supplied by Nanjing Healthline Medical Technology Co., Ltd, Nanjing, China. Ethanol, isopropanol and n-hexane were purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd, Shanghai, China. Diethyl amine (DEA) was purchased from Aladdin Reagent Co., Ltd, Shanghai, China. Methyl alcohol was purchased from Merck Ltd, Darmstadt, Germany. All the reagents used were of HPLC grade.

HPLC conditions

An Agilent 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with G1379A degasser, G1311A quaternary gradient pump, G1329A auto sampler, G1316A column thermostat and G1315B diode array detector. The separation was performed on a Chiralpak AY-H column (250 x 4.6 mm i.d., 5 μm particle size; Daicel, Tokyo, Japan) at 35°C. A mobile phase used was a combination of n-hexane : ethanol : DEA (55 : 45 : 0.1, v/v/v) with the flow rate at 1.0 mL/min. All mobile phases prepared were filtered through a 0.45-μm filter membrane and degassed with an ultrasonic bath before use. The detection wavelength was set at 254 nm, and the injection volume was 20 μL.

Preparation of assay and parameter analysis

All samples were dissolved in the mixed solutions of methyl alcohol and ethyl alcohol (1 : 1, v/v). The void time (t0) was determined by the retention of 1,3,5-tri-tert-butylibenzene. Capacity factor (k') was equal to (tR − t0)/t0, where tR is the retention of the analytes. A separation factor (α) was calculated from the equation of k'1/k'2, where k'1 and k'2 are the capacity factors of first and second eluted analytes, respectively.

The relationships between chromatographic profiles and column temperature can be described by the Van’t Hoff equation of

\[ \frac{k'}{k''} = \frac{1}{1 + \frac{(T_0 - T) \cdot \Delta V}{R \cdot T}} \]

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where \( R \) is the universal gas constant, \( T \) is the column temperature and \( \varphi \) is the phase ratio. \( \Delta H^0 \) and \( \Delta S^0 \) stand for the differences in the enthalpy and entropy when one enantiomer transfers from mobile phase to the stationary phase. \( \Delta H^0 \) and \( \Delta S^0 \) are the differences in \( \Delta f^0 \) and \( \Delta S^0 \) for a given enantiomeric pair. \( \Delta S^\varphi \) is introduced to replace the expression of \( \frac{\Delta S^0}{R} + \ln \varphi \) and \( \Delta S^\varphi \) could not be used to determine the value of \( \Delta S^0 \) for \( \varphi \) is unknown. If \( \ln k' \) or \( \ln \alpha \) versus \( 1/T \) shows good linearity under studied temperature, the related thermodynamic parameters, namely \( \Delta f^0 \), \( \Delta f^\varphi \), \( \Delta S^\varphi \) and \( \Delta S^0 \), can be deduced from plots' slope and intercept, and those data may also be used to explain the strength of the interactions between the analytes and CSPs.

**Results**

**Method validation**

It is important to establish a simple and rapid method used for routine quality control of mirabegron enantiomers (15). The proposed HPLC method was validated according to the ICH guidelines and was found to comply (16).

**System suitability**

The system suitability was evaluated by the analysis of the standard solutions containing MI-I and II in sextuplicate. The typical HPLC chromatogram is shown in Figure 2, where the enantiomers achieved baseline separation as the resolution was 3.69. The symmetry factor and theoretical plate number for MI-I were \(~0.91\) and 2884, respectively, and the values for MI-II were 0.99 and 2281, respectively.

**Linearity**

A series of MI-II solutions were prepared at the concentration ranging from LOQ to 1% level spiked to the MI-I samples, and the concentrations for MI-I samples were varied from 50 to 120% of the routine detecting concentration (0.5 mg/mL). Excellent linear equations were obtained from the peak area response (y) to the concentration (x) of the analytes using least squares linear regression, and the correlation coefficient (r) was greater than 0.9993.

**Limits of detection and quantification**

The LOD and LOQ levels were determined at the peak signal-to-noise ratio of about 3 and 10, respectively. By injecting a series of dilutions with known concentrations, the profiles of LOD and LOQ were summarized in Table I.

**Precision and accuracy**

The intra- and interday precisions were investigated from six replicate sample solutions (\( \mu \)g/mL) on the same day and three consecutive days, respectively. The intraday precision (relative standard deviation, RSD) was <1.65%, and interday precision of within 1.75% (RSD). Meanwhile, the accuracy was evaluated by the recovery experiments at three concentration levels (0.25, 0.5 and 0.75%) spiked to the MI-I samples in triplicate, and all recoveries were within 98–102%, RSD <2%. The above data indicated good precision and accuracy of the method.

**Robustness and stability**

Small changes in column temperature (35 ± 2°C), flow rate (1.0 ± 0.05 mL/min), composition of mobile phase (ethanol, 45 ± 1%) and the additive (DEA, 0.1 ± 0.01%) were assessed, and all the deliberate changes did not alter the elution behavior significantly. The resolution between MI-I and II was greater than 3.5. The symmetry factor was within 0.88–1.01 and the number of theoretical plates was >2000. These data illustrated the robustness of the proposed method sufficiently. The sample solutions were tested at 2 h intervals, and the RSD (%) of the peak area response was <1.85% over 0–10 h, which revealed that sample solutions were stable up to 10 h.

**Evaluation of thermodynamic parameter**

A variable temperature study was performed over the range of 20–40°C with an interval of 5°C, and all of the above experiments got baseline resolution between two analytes. As presented in Table II, improved resolution and peak shapes were obtained at relatively high column temperature, while the values of \( k' \) and \( \alpha \) decreased with an increasing temperature.
Table I
The Method Validation Profiles of Mirabegron Enantiomers

<table>
<thead>
<tr>
<th>Enantiomers</th>
<th>Range (µg/mL)</th>
<th>Regression equation</th>
<th>$R^2$</th>
<th>LOQ (ng)</th>
<th>LOQ (ng)</th>
<th>Recovery (%)</th>
<th>Intraday precision</th>
<th>Interday precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>251.4–603.2</td>
<td>$y = 52.85x + 0.137$</td>
<td>0.994</td>
<td>0.73</td>
<td>2.43</td>
<td>—</td>
<td>1.62</td>
<td>1.16</td>
</tr>
<tr>
<td>II</td>
<td>0.129–5.190</td>
<td>$y = 50.51x + 0.075$</td>
<td>0.998</td>
<td>0.77</td>
<td>2.58</td>
<td>99.2, 1.08</td>
<td>1.43</td>
<td>1.73</td>
</tr>
</tbody>
</table>

*Mean recovery of the analyte in three different concentrations.

Table II
Effects of Ethanol Content and Column Temperature on Chromatographic Parameters

<table>
<thead>
<tr>
<th>Ethanol (%, v/v)</th>
<th>Column temperature (°C)</th>
<th>Capacity factor $k'_I$</th>
<th>Capacity factor $k'_II$</th>
<th>Separation factor $α$</th>
<th>Resolution $Rs$</th>
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<tr>
<td>30</td>
<td>20</td>
<td>4.03</td>
<td>6.64</td>
<td>1.65</td>
<td>4.56</td>
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<tr>
<td></td>
<td>25</td>
<td>3.94</td>
<td>6.46</td>
<td>1.64</td>
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<td>1.47</td>
<td>2.33</td>
<td>1.59</td>
<td>3.69</td>
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</table>

In the present study, the enantiomeric pair of mirabegron all gave highly linear Van’t Hoff plots (Figure 3, regression coefficients $R > 0.99$), reflecting the conformation of CSPs did not change under the test conditions, nor did the selectivity and retention mechanisms (17). From the derived thermodynamic parameters (Table III), the absolute $\Delta H^0_0$ and $\Delta S^0_0$ values decreased as ethanol content varying from 30 to 45%, indicating the interactions between analytes and CSPs decreased with the increasing ethanol content.

The values for $\Delta S^0$ and $\Delta S^0_0$ were negative, suggesting that the chiral separation was enthalpy driven. MI-II, the later-eluting analyte, represented stronger interactions with CSPs than MI-I. Overall, the molecules become ordered during the adsorption process. The separation factor values decreased with the increasing column temperature.

**Discussion**

**Method optimization**

To develop a suitable HPLC method for separation of the enantiomers, different columns and mobile phases were employed. A mixture solution of MI-I and II were used during the whole method development. The widely used traditional Chiralpak AD-H, a chiral column coated with amyllose tris-(3,5-dimethylphenylcarbamate), was initially selected; n-hexane and different organic modifiers (isopropanol and ethanol) were tried as a mobile phase. Results revealed that when the content of organic modifiers was < 30%, the analytes were hard to be eluted, and badly tailing peaks were exhibited. When the content of organic modifiers improved, no hint of baseline resolution between the enantiomers was obtained in spite of adjusting the composition of n-hexane, isopropanol and ethanol in a mobile phase. Peaks of the analytes were even overlapped under n-hexane–ethanol (60 : 40) as a mobile phase. Then, Chiralpak AY-H, a column coated with amyllose tris-(5-chloro-2-methylphenylcarbamate), was studied under normal phase conditions. It is proved to have a relatively well resolution between two enantiomers when using n-hexane and ethanol as mobile phases, and the peak shapes were also symmetrical. It may be due to the relatively high-polarity and unique molecular space structure, ethanol demonstrated better elution ability than isopropanol and the retention time of analytes delayed significantly when moving from ethanol to isopropanol.

Compared the results obtained on Chiralpak AD-H and Chiralpak AY-H, the two columns were all not suitable for separation of mirabegron enantiomers under the n-hexane and isopropanol system. While under the n-hexane and ethanol system, the chiral recognition ability of Chiralpak AY-H column improved significantly and was far better than Chiralpak AD-H. So, Chiralpak AY-H column was supposed to be a good choice for separation of mirabegron enantiomers. In consideration of the weak alkalinity of mirabegron, the addition of DEA was introduced. More symmetrical and narrow peak shapes were achieved with the presence of 0.1% DEA in a mobile phase. Therefore, the optimized condition for mirabegron enantioseparation was studied on a Chiralpak AY-H column with n-hexane, ethanol and DEA as a mobile phase.

**Evaluation of enantioseparation mechanisms**

In general, the enantioseparation mechanisms of amyllose CSPs are dependent on the highly ordered spiral cavum structure formed by amyllose, as well as the functional groups on the substituent. Once the guest molecules entered into the spiral cavum (chiral cavity), they formed as inclusion complexes with CSPs. Meanwhile, the functional groups provided different intermolecular forces with analytes and CSPs for the successful enantioseparation (18).

Chiralpak AD-H and AY-H are chiral columns coated with amyllose tris-(3,5-dimethylphenylcarbamate) and amyllose tris-(5-chloro-2-methylphenylcarbamate), respectively. For the Chiralpak AD-H column, the function groups, such as C=O and N–H, could form hydrogen bond interactions; carbamyl groups could form a dipolar interaction; and benzene ring could form π–π conjugate actions. For the Chiralpak AY-H column, apart from the...
function groups described in above, the chlorine atom with strong electronegativity is introduced. It increases the action sites with analytes (19), but also improves the dipolar interactions by induction effects. Maybe due to the halogenated CSPs, Chiralpak AY-H column represented better chiral selectivity than AD-H for mirabegron enantiomers.

Effect of ethanol (%) in a mobile phase
By altering the proportion of ethanol in a mobile phase, the effect of separation on mirabegron and its enantiomer was evaluated. The chromatographic retention time decreased sharply with the proportion of ethanol ranging from 30 to 45%. It might be due to decreased hydrogen bond interactions between analytes and CSPs as the ethanol content increased in a mobile phase. Overall, when column temperature maintained at 20–40°C, the capacity factors ($k'$), separation factors ($\alpha$) and resolution (Rs) decreased with the increasing ethanol content.

Conclusions
A simple and sensitive normal phase HPLC method was developed and validated to separate mirabegron enantiomers on a Chiralpak AY-H column. A better elution was obtained using ethanol than isopropanol as organic modifiers. The $\ln k'$ and $1/T$ were linear under different ethanol content (30–45%) conditions, reflecting the conformation of CSPs and the retention mechanisms kept unchanged in the temperature range of 20–40°C. The negative apparent thermodynamic parameters indicated that the separation was driven by enthalpy. The optimized method could not only be used to routine analysis of mirabegron enantiomers with HPLC, but also be compatible with LC–MS-MS.

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Conflict of interest statement
The authors alone are responsible for the content and writing of this paper.

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