Condition Optimization for The Analysis of Risperidone and 9-OH-Risperidone by High-Performance Liquid Chromatography

Luki Yogaswara Yusuf1,4, *Aliya Nur Hasanah1, Melisa Intan Barliana2,3

1Department of Pharmaceutical Analysis and Medicinal Chemistry, Faculty of Pharmacy, Padjadjaran University, Jl. Raya Bandung Sumedang KM 21, Jatinangor, Sumedang, West Java, Indonesia 2Department of Biological Pharmacy, Faculty of Pharmacy, Padjadjaran University, Jl. Raya Bandung Sumedang KM 21, Jatinangor, Sumedang, West Java, Indonesia 3Center of Excellence in Higher Education for Pharmaceutical Care Innovation, Padjadjaran University, Bandung, Indonesia 4Department of Pharmaceutical Chemistry Analysis, Faculty of Pharmacy, Jenderal Achmad Yani University, Cimahi, Indonesia. *Email: aliya.n.hasanah@unpad.ac.id

DOI: 10.31964/mltj.v0i0.424

Abstract: Risperidone (RIS) is one of the most widely used atypical antipsychotics for treating schizophrenia in hospitals. RIS is metabolized by the liver and produces the primary active metabolite 9-OH-Risperidone (9-OHR). In the process of RIS metabolism, it is suspected that there are gene polymorphisms that cause variations in patient responses. Analysis of RIS and 9-OHR levels in the patient's blood can help to explain the various responses. High-Performance Liquid Chromatography (HPLC) is the most popular method to analyze RIS and 9-OHR, but many deficiencies were found in the chromatograms in the previous study. This research aims to obtain optimal conditions of the analysis prior to method validation. Condition optimization by optimizing the wavelength, composition of the mobile phase, pH, flow rate, and particle size of the stationary phase. The results showed that the wavelength was 279 nm, the mobile phase was 0.05 M KH2PO4 pH 3.7: acetonitrile (94:6, v/v) plus 0.3% triethylamine, and the flow rate was 1.2 ml/min in the stationary phase (LiChroCART® RP 18; 250x4 mm; 10 µm) being the optimal condition. This method is suggested to continue method validation for analyzing RIS and 9-OHR in the serum or plasma.

Keywords: Risperidone; 9-OH-risperidone; clozapine; High-Performance Liquid Chromatography

INTRODUCTION

Schizophrenia is a chronic mental disorder and characterized by positive symptoms (hallucinations, delusions, thought disorders), negative (social withdrawal or social withdrawal), and functional and cognitive disorders (Zhang & Malhotra, 2011). RIS is an atypical antipsychotic drug used to treat schizophrenia, especially in hospitals. RIS is metabolized in the liver by the cytochrome P450 enzymes CYP3A4 and CYP2D to produce the essential active metabolite (9-OHR), which has the same pharmacological activity (Patients, Weide, & Weide, 2015). However, some patients are not responsive to the treatment, so trial and error occur in treating schizophrenia (Zhang et al., 2013).

One of the causes of varying therapeutic responses is the influence of gene polymorphisms in drug metabolism and affects drug pharmacokinetics (Ackenheil and Weber, 2004), so monitoring of drug therapy in serum or plasma is needed to help explain drug unresponsiveness (Lisbeth et al., 2016). Determination of RIS and 9-OHR levels in the schizophrenic blood patients need to use analytical methods that

Corresponding Author: Aliya Nur Hasanah
Department of Pharmaceutical Analysis and Medicinal Chemistry, Faculty of Pharmacy, Padjadjaran University, Jl. Raya Bandung Sumedang KM 21, Jatinangor, Sumedang, Jawa Barat, Indonesia.
Email: aliya.n.hasanah@unpad.ac.id
available at the hospital, a simple and easy method because it will be carried out routinely in hospitals but still provides a good validation value so it can be a reference for the following therapeutic step for schizophrenic patients.

Many studies have been carried out to analyze RIS and 9-OHR in blood, especially using HPLC with a detection limit value in the range of therapeutic levels, but the accuracy value obtained is too high above 120% 9-OHR. The correlation coefficient value of the calibration curve is too low at 0.97 for the RIS (Titier, Deridet, Cardone, Abouelfath, & Moore, 2002). Other studies also found deficiencies in the chromogram, with low resolution and high tailing factor on the RIS and 9-OHR (Salemi, Spina, Avenoso, & Facciola, 2000). Studies (Kirschbaum et al., 2008) also took more than 20 minutes run time to separate RIS, 9-OHR and Clozapine (IS).

Based on the above background, the purpose of this research is to obtain the optimal condition for the analysis of RIS and 9-OHR before method validation analyzing their levels in serum or plasma. Optimization is carried out, including changes in wavelength, the composition of the mobile phase, pH, flow rate, and particle size of the stationary phase in the column.

MATERIALS AND METHODS
Tools and materials
The tools used in this study were UV-Vis Spectrophotometer (Shimadzu), HPLC (Shimadzu LC-10AT), column 1 (LiChroCART® RP 18; 250x4 mm; 5 m), column 2 (LiChroCART® RP 18; 250x4 mm; 10 m), pH meter (Mettler Toledo), ultrasonicator (Ney), analytical balance (Ohaus), vacuum pump (Rocker 300), 0.45 m filter (Sartorius), micropipette (Socorex) and glassware commonly used in analytical laboratories.

The materials used in this study were risperidone (Tokyo Chemical Industries/ TCI, analytical grade), 9-OH-risperidone (TCI, analytical grade), clozapine (IS) (TCI, analytical grade) as internal standard, methanol (Merck, analytical grade), acetonitrile (Merck, analytical grade), NaH2PO4 (Merck, analytical grade), Na2HPO4 (Merck, analytical grade), KH2PO4 (Merck, analytical grade), orthophosphoric acid (Merck, analytical grade), triethylamine ( Sigma Aldrich, analytical grade) and sterile aquabidest (IPHA, analytical grade).

Determination of maximum wavelength RIS and 9-OHR
Each 10 mg RIS, 9-OHR, and IS standard was dissolved in 10 mL of methanol, then diluted again with aquabidest to obtain a concentration of 10 g/mL each. The absorbance of RIS, 9-OHR, and IS standard solutions were measured respectively on a UV-Vis spectrophotometer with a wavelength range of 200 nm – 400 nm.

Mobile phase 1 (0.1 M phosphate buffer pH 3.8: acetonitrile)
Mixed standard solution RIS, 9-OHR ten µg/mL and IS 1 µg/mL is made by dissolving each of 0.1 mL RIS and 9-OHR standard solution (1000 g/mL) and 1 mL IS solution (10 g/mL) until 10 mL with aquabidest. Orthofosforic acid solution (1:10) to obtain pH 3.8 in the mobile phase is made from 1 mL of orthophosphoric acid P. dissolve until 10 mL with aquabidest.

Mobile phase 0.1 M phosphate buffer pH 3,8 made by dissolving 2.65 g Na2HPO4 in 100 mL aquabidest and 1.77 g NaH2PO4 in 100 mL aquabidest. Na2HPO4 solution and the NaH2PO4 solution were mixed with 100 mL of aquabidest and added until a pH of 3.8 was obtained by orthophosphoric acid (1:10). The phosphate buffer solution was then filtered through a 0.45 µm filter using a vacuum and sonicated using a sonicator for 15 minutes.
Mobile phase 2 (0.05 M KH₂PO₄ pH 3.7: acetonitrile)

Orthofosforic acid (25%) to obtain pH 3.7 in the mobile phase and pH 2.2 in the solvent is made by dissolving 2.5 mL orthofosforic P. until 10 ml with aquabidest. 0.1 M KH₂PO₄ pH 2.2 solvent is made by dissolving 1.36 gr KH₂PO₄ with 100 mL aquabidest, and orthofosforic acid 25% was added until pH 2.2. Mixed standard solution RIS, 9-OHR, and IS 10 µg/mL is made by dissolving each of 0.1 mL RIS, 9-OHR standard solution, and IS (1000 µg/mL) with 0.1 M KH₂PO₄ pH 2.2 until 10 ml aquabidest.

0.05 M KH₂PO₄ pH 3.7 mobile phase solution is made by dissolving 1.36 gr KH₂PO₄ until 200 mL with aquabidest and added with 0.3 % triethylamine. 25% orthofosforic was added until pH 3.7 was obtained. KH₂PO₄ solution was then filtered through a 0.45 µm filter using a vacuum and sonicated using a sonicator for 15 minutes.

Analysis optimization

Optimization of the analytical conditions was carried out using three conditions. 20 µL of the mixed standard solution was injected into the HPLC system with different compositions of mobile phase 1 and 2, pH, analyte solvent, flow rate, column, and wavelength (Table 1).

Table 1. Condition Optimization Analysis

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mobile phase composition</th>
<th>Flow rate (ml/min)</th>
<th>Wavelength (nm)</th>
<th>Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1. 0,1 M phosphate buffer pH acetonitrile</td>
<td>43</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1. 0,1 M phosphate buffer pH acetonitrile</td>
<td>40</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2. 0,05 M KH₂PO₄ pH acetonitrile</td>
<td>70</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>93</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Determination of maximum wavelength RIS and 9-OHR

UV-Vis absorption spectra of RIS solution (Figure 1. A), 9-OHR (Figure 1. B), and IS (Figure 1. C) in methanol obtained the maximum wavelength at (236 and 279 nm), (237 and 279 nm), and 215 nm. Previous studies used a wavelength of 230-240 nm (Frahnert, Rao, & Grasmäder, 2003; Mandrioli, Mercolini, Lateana, Boncompagni, & Augusta, 2011; Raggi, Bugamelli, Sabbioni, Saracino, & Petio, 2005) and at a wavelength of 277-285 nm (Berecz, Dorado, & Caceres, 2003; Hplc-uv, 2018;
Kirschbaum et al., 2008; Naumovska et al., 2017; Salemi et al., 2000; Selva & Ramanathan, 2016; Titter et al., 2002).

The maximum wavelengths used in this optimization are 236 nm and 279 nm. At both wavelengths, RIS and 9-OHR have the most significant absorption so that when measurements with low concentrations in serum can still be adequately analyzed and are still provide good absorption.

![Figure 1. UV-Vis Absorption Spectra (A) RIS, (B) 9-OHR and (C) IS](image)

**Condition optimization 1 and 2**

Mobile phase 1 analysis with an initial composition ratio of 43:57 (figure 2. A) shows that the first peak was owned by a blank with a retention time of 2.119 minutes as obtained during the blank analysis and the singular form of the three analytes. The second chromatogram peak was owned by 9-OHR (1) with a retention time of 2.718 minutes, and the third chromatogram peak was owned by the combination of RIS-IS (2) with a retention time 3.253 minutes. Phosphate buffer addition at the composition of (55:45) (Figure 2.D) shows a further separation between the 9-OHR peak (1) with a retention time of 3.269 minutes and the RIS-IS peak (2) with a retention time of 4.941 minutes. In this condition of mobile phase 1, the increase in the amount of phosphate buffer can only give a change in a longer retention time and more excellent resolution but has not been able to separate the peaks of the RIS and IS chromatograms. To determine the effect of the pH in the mobile phase, the pH was changed to 2.9 in the following condition.

In the comparison of the same composition (55:45) (Figure 3.D) compared to (Figure 2.D), the retention time is faster for pH 2.9 (9-OHR 3,223 minutes and RIS+IS 4,361 minutes) while for pH 3.8 (9-OHR 3,269 minutes and RIS+IS 4,941 minutes). The lower change in the pH of the mobile phase was able to accelerate the retention time, but the chromatograms produced were almost the same and were still unable to separate the RIS and IS peaks.

The inability of the mobile phase to separate RIS and IS chromatograms could be caused by the concentration of IS being too low in the mixture (1 µg/mL). However, after increasing the concentration to 10 µg/mL, it was still unable to separate them. In the following condition, the type of phosphate buffer was changed in the mobile phase, column, and the analyte solvent under the following conditions without changing the pH.

**Condition Optimization 3**

Mobile phase 2 analysis with a composition ratio (70:30)(Figure 4. A) shows RIS peak is joining IS peak and almost same as separation by mobile phase 1, but at composition ratio (80:20)(Figure 4. B) had a better peak for the separation RIS and IS. Compared to the mobile phase 1, especially at the composition ratio of 90:10 (Figure
4. C), the three analytes separated with retention times of 9-OHR (1) 5.8 minutes, RIS (2) 10,769 minutes and IS (3) 13,504 minutes, although the resolution for the chromatogram peak RIS (2) and CLO (3) did not look good enough. Mobile phase changes in the composition ratio indicate that the greater the composition of KH2PO4, the retention time will be longer for the three analytes. However, the resolution of RIS and IS chromatograms is getting better. An increase in the amount of KH2PO4 is required. However, the results will take a longer time.

![Figure 2. Chromatogram with Mobile Phase 0.1 M Phosphate Buffer pH 3.8: Acetonitrile (A) 43:57, (B) 45:55, (C) 50:50, (D) 55:45, (1) 9-OHR Peak, (2) RIS-IS Peak](image)

![Figure 3. Chromatogram with Mobile Phase 0.1 M Phosphate Buffer pH 2.9: Acetonitrile (A) 40:60 (B) 45:55 (C) 50:50 (D) 55:45, (1) 9-OHR Peak (2) RIS+IS Peak](image)

The use of analyte solvent 0.1 M KH2PO4 pH 2.2, which is similar to the mobile phase, was able to help separate the analytes optimally. This is because the pH of the solvent helps prevent ionization of the three analytes during separation. 0.3% triethylamine hat added to the mobile phase is also able to suppress ionization and helps to improve the chromatogram peaks of each analyte under ideal conditions.
Table 2. Optimization Mobile Phase and Flow Rate Result

<table>
<thead>
<tr>
<th>Composition</th>
<th>Flow rate ml/ min</th>
<th>Chromatogram</th>
<th>Retention time</th>
<th>Resolution</th>
<th>Tailing Factor</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acn: (8:92) KH₂PO₄</td>
<td>0.8</td>
<td>9-OHR</td>
<td>8,641</td>
<td>1,507</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acn: (7:93) KH₂PO₄</td>
<td>1</td>
<td>9-OHR</td>
<td>7,822</td>
<td>1,416</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acn: (6:94) KH₂PO₄</td>
<td>1.2</td>
<td>9-OHR</td>
<td>7,294</td>
<td>1,417</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acn: (5:95) KH₂PO₄</td>
<td>1.4</td>
<td>9-OHR</td>
<td>7,324</td>
<td>1,418</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Further analysis used mobile phase 2 with compositional ratios; flow rate ((8:92): 0.8, (7:93): 1, (6:94): 1.2, (5:95): 1.4), sequentially (Fig. 5. A, 5. B, 5. C, 5. D) shows the same chromatogram but the fastest retention time is owned by the ratio of the composition of the mobile phase; flow rate (6:94; 1.2 ml/minute)(Figure 5. C) with the retention time of 9-OHR 7,294 minutes, RIS 13,644 minutes and IS 18,776 minutes. The resolution value of the four conditions was in the range of ≥1.5 (Snyder,
Kirkland, & Glajch, 1997), and the highest resolution value was obtained from the composition (5:95; 1.4 ml/min)(Figure 5.D) at the peak of 9-OHR and RIS of 2.791, however the peak tailings factor value of RIS in this composition is too large (1.731) when compared to the composition (6:94; 1.2 ml/min) of 1.537. However, the tailing factor values of the four analytes are in the range <2 (Snyder et al., 1997) and meet the requirements the condition (6:94; 1.2 ml/min) has the smallest tailings factor value, which is 1.462 on average so that it becomes optimum condition value. Based on the retention time, resolution, and tailing factor, the condition (6:94; 1.2 ml/min) became the optimum condition. The optimization results can be seen in Table 2.

Compared with previous studies (Salemi et al., 2000), the retention time in this study was not better due to the differences in the columns used. Previous studies used a size (100x4.6 mm, 3 µm), 150 mm shorter, and a particle size of 7 µm smaller to reduce retention time, but in this study, the resolution and tailings factor got better values. On the other hand, compared with (Kirschbaum et al., 2008), this study was better in the run time because the method needed less than 20 minutes to finish the separation for RIS, 9-OHR, and IS.

This optimal condition benefited better run time, resolution, and tailing factor, so it can be continued to the validation stage to analyze RIS and 9-OHR in the blood. The limitation of this research is that in the mobile phase, the majority consists of phosphate buffer that could clog the column, so it needs routinely cleaned to prevent colon damage. This optimal condition has also become the promising method routinely used in hospitals to improve the quality of schizophrenia medication.

CONCLUSION
Condition optimization analysis of RIS and 9-OHR with internal standard (CLO) on Shimadzu LC-10AV column LiChroCART® RP 18 (250x4 mm;10 µm) was obtained at mobile phase 0.05 M KH2PO4 pH 3.7: acetonitrile (94:6 v/v), 0.3% triethylamine, at the wavelength of 279 nm and a flow rate of 1.2 ml/min. This method is suggested to continue method validation for analyzing RIS and 9-OHR in the serum or plasma.

ACKNOWLEDGEMENT
The authors would like to thank Padjadjaran University for the research funding in this study.

CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest regarding the publication of this paper.

REFERENCES


