Liquid Chromatography-Tandem Mass Spectrometry Analysis of Riboflavin in Beagle Dog Plasma for Pharmacokinetic Studies

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Abstract: Riboflavin is a water-soluble vitamin, which serves as a precursor to flavin mononucleotide and flavin adenine dinucleotide. This study aimed to develop a simple and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis for the quantification of riboflavin in the Beagle dog plasma. This method utilized simple protein precipitation with acetonitrile and 13C4-15N2-riboflavin was used as an internal standard (IS). For chromatographic separation, a hydrophilic interaction liquid chromatography (HILIC) column was used with gradient elution. The mobile phase consisted of 0.1% (v/v) aqueous formic acid with 10 mM ammonium formate and acetonitrile with 0.1% (v/v) formic acid. Since riboflavin is an endogenous compound, 4% bovine serum albumin in phosphate buffered saline was used as a surrogate matrix to prepare the calibration curve. The quantification limit for riboflavin in the Beagle dog plasma was 5 ng/mL. The method was fully validated for its specificity, sensitivity, accuracy and precision, recovery, and stability according to the US FDA guidance. The developed LC-MS/MS method may be useful for the in vivo pharmacokinetic studies of riboflavin.

Keywords: riboflavin, LC-MS/MS, validation, plasma, Beagle dog

Introduction

Riboflavin (Vitamin B2), as a precursor to flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), is essential nutrition for mammals.1 Riboflavin also plays a vital role in mitochondrial energy metabolism, and the metabolism of fats, ketone bodies, carbohydrates, and proteins.2 Riboflavin deficiency may appear in the people who long-term depart from standard nutritional supplementation, including sailors, pathfinders, soldiers, or people in poverty. The associated symptoms to riboflavin deficiency include cracked and red lips, inflammation of the lining of mouth and tongue, mouth ulcers, cracks at the corners of the mouth, a sore throat, dry and scaling skin, fluid in the mucous membranes, iron-deficiency anemia, and the oral-ocular–genital syndrome.3

After oral ingestion, riboflavin is mostly absorbed in the upper duodenal area by an active transport mechanism, while absorption in the lower intestine is minimal.4,5 Thus, massive doses of multivitamins may have marginal benefits with respect to riboflavin, as the unabsorbed amounts would be catabolized by the microbial flora or excreted via the stool.1 On the other hand, gastroretentive systems have shown their potential to improve bioavailability of riboflavin, which is related to its narrow absorption window in the upper part of gastrointestinal tract.6,7 As the absorption window of riboflavin has been widely known, many investigators have attempted to develop gastroretentive systems by using riboflavin as a model drug.

Several analytical methods such as high-performance liquid chromatography (HPLC) with UV-Vis spectrometry,8 UV detection,9 and fluorescence determination10 have been reported for the determination of riboflavin in plasma. These methods usually involve more sample processing steps and long execution time. Although an isotope dilution method using liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been recently reported,11 there are limited analytical methods using LC-MS/MS for riboflavin that can be utilized for pharmacokinetic studies. Therefore, this study aimed to develop an LC-MS/MS method with high sensitivity to determine riboflavin in the dog plasma. The developed analytical method may be useful for the in vivo pharmacokinetic evaluation of riboflavin.
Experimental

Material
Riboflavin was obtained from Korea United pharm. INC. (Seoul, Korea). Lactose (Avicel®) was purchased from Meggle GmbH (Wasserburg, Germany). Hydroxypropyl methylcellulose (METHOLOSE® 90SH) was obtained from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Dimethyl sulfoxide was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and magnesium stearate was purchased from Merck Co. (Darmstadt, Germany). Sodium hydroxide and hydrochloric acid was purchased from Faci Asia Pacific Pte Ltd. (Jurong Island, Singapore). A phosphate monobasic was purchased from Daejung Chemicals & Metals co., Ltd. (Gyeonggi-do, Korea). Potassium sulfoxide was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dimethyl grade acetonitrile, methanol and water were purchased from J.T. Baker Co. (Philipsburg, NJ).

Instruments and LC-MS/MS analysis conditions
LC-MS/MS analysis was performed by an Agilent 6490 triple-quadrupole mass spectrometer coupled with Agilent 1260 HPLC (Agilent, Santa Clara, CA, USA). Compounds were separated on Atlantis HILIC Silica column (150 × 2.1 mm, i.d., 3 μm, Waters, Milford, MA, USA) with a SecurityGuard Cartridge Kit (Phenomenex, Torrance, CA, USA). Chromatographic separations were performed by using a binary gradient mobile phase composed of mobile phase A (0.1% formic acid and 10 mM ammonium formate in distilled water) and mobile phase B (0.1% formic acid in acetonitrile) and composition and flow are shown in Table 1. The column oven temperature was set at 30°C and the total run time was 15.0 min.

The ESI source was operated in positive mode and the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with a dwell time of 200 ms per MRM channel. Gas temperature, gas flow rate, and nebulizer gas pressure were set at 180°C, 11 L/min, and 40 psi, respectively. The selected precursor/product ion pairs were m/z 377.1 → 243.1 for riboflavin, m/z 383.1 → 248.8 for \(^{13}\text{C}_4\text{N}_2\)-riboflavin (IS). The collision energy was 25 V for both riboflavin and IS. The mass spectrometric data were processed by Mass Hunter Quantitative Analysis (Agilent Technologies, Santa Clara, CA, USA).

Sample preparation
The stock solutions of riboflavin (1 mg/mL) and IS (\(^{13}\text{C}_4\text{N}_2\)-riboflavin, 1 mg/mL) were prepared by dissolving each substance in DMSO and stored at -20°C. Calibration standard samples of riboflavin were prepared by diluting the stock solution with distilled water followed by spiking in the surrogate blank serum (4% bovine serum albumin in phosphate-buffered saline) to obtain the following concentrations: 5, 10, 25, 50, 100, 250, and 500 ng/mL. Quality control (QC) samples were prepared by diluting the stock solution with water and spiking in blank plasma to obtain the 5 (LLOQ), 8 (LQC), 200 (MQC), and 400 ng/mL (HQC). All calibration standard samples and QC samples were stored at -20°C. Working solution of IS (250 ng/mL) was prepared by diluting the stock solution with distilled water and stored at 4°C.

For sample preparation, an aliquot (50 μL) of standard, QC, or Beagle dog plasma samples was first mixed with 50 μL of working IS solution (250 ng/mL). Then, the mixture was vortex-mixed with 200 μL acetonitrile, which was used as a protein precipitation agent, for 30 s. Following centrifugation for 10 min at 4,000 × g, 50 μL of the supernatant was transferred to a polypropylene HPLC vial with 100 μL mobile phase B. The mixture was vortex-mixed for 30 s and a portion (10 μL) was injected into the LC-MS/MS.

Assay validation
Equivalency between surrogate and real dog plasma
Since riboflavin is an endogenous compound, a surrogate matrix was used to prepare standard solutions instead of real dog plasma. To test the equivalency between surrogate matrix and real dog plasma, QC samples at three concentration levels were prepared in dog plasma. The equivalency was calculated as:

\[
\text{Equivalency(\%)} = \frac{\text{Response}_{\text{plasma spike}} - \text{Response}_{\text{plasma blank}}}{\text{Response}_{\text{surrogate spike}}} \times 100\% 
\]

where Response_{plasma spike} is the peak area obtained from riboflavin-spiked QC sample in dog plasma, Response_{plasma blank} is the peak area obtained from riboflavin in blank dog plasma, and Response_{surrogate spike} is the peak area obtained from riboflavin-spiked QC sample in surrogate matrix. Experiments were conducted in triplicate.
Process efficiency

To evaluate the extraction recovery and matrix effect, the QC samples at three concentration levels were prepared in surrogate matrix. The process efficiency was calculated as:

\[
\text{Process efficiency} \% = \frac{\text{Response}_{\text{surrogate spike}}}{\text{Response}_{\text{matrix-free spike}}} \times 100\%
\]

where \(\text{Response}_{\text{surrogate spike}}\) is the peak area obtained from riboflavin-spiked QC sample in surrogate matrix and \(\text{Response}_{\text{matrix-free spike}}\) is the peak area obtained from the same concentration in distilled water. Experiments were conducted in triplicate.

Specificity, sensitivity, and linearity

To examine the potential presence of endogenous and exogenous substance in the surrogate matrix, endogenous or exogenous interferences were assessed by comparing the surrogate blank matrix and the surrogate matrix spiked with analytes and IS. The interference of matrix for analyte was calculated as:

\[
\text{Interference of matrix} \% = \frac{\text{Analyte response}_{\text{blank}}}{\text{Analyte response}_{\text{LLOQ spike}}} \times 100\%
\]

where \(\text{Analyte response}_{\text{blank}}\) is the peak area obtained from blank sample in surrogate matrix and \(\text{Analyte response}_{\text{LLOQ spike}}\) is the peak area obtained from riboflavin-spiked sample at LLOQ concentration (5 ng/mL) in surrogate matrix. Experiments were conducted in triplicate. The interference of IS for analyte was calculated as:

\[
\text{Interference of IS} \% = \frac{\text{Analyte response}_{\text{IS spike}}}{\text{Analyte response}_{\text{LLOQ spike}}} \times 100\%
\]

where \(\text{Analyte response}_{\text{IS spike}}\) is the peak area obtained from IS-spiked sample (250 ng/mL) in surrogate matrix and \(\text{Analyte response}_{\text{LLOQ spike}}\) is the peak area obtained from riboflavin-spiked sample at LLOQ concentration (5 ng/mL) in surrogate matrix. Experiments were conducted in triplicate.

The LLOQ was determined by analyzing 5 replicates of the lowest standard on the calibration curve that yielded a signal-to-noise (S/N) ratio > 10, with acceptable accuracy and precision (< 20%).

Calibration curves in the surrogate matrix were created by plotting the peak area ratio of riboflavin to IS against the nominal concentrations of the riboflavin. The weighted linear regression method (1/x) was applied and acceptable correlation coefficients were set as 0.999 or greater.

Accuracy and precision

Accuracy and precision of the LC-MS/MS method were determined by analyzing the QC samples at LLOQ, low, medium, and high QC concentrations on the same day (intra-day) and three consecutive days (inter-day). Intra-day and inter-day accuracy and precision were assessed in quintuplicate and triplicate, respectively.

The accuracy was expressed as the percentage of the mean back-calculated concentration versus nominal concentration. The precision was expressed as the coefficient of variation of each concentration. Acceptable criteria for accuracy and precision were within ± 15% relative error from the theoretical values and within ± 15% relative standard deviation except at the LLOQ where it should not deviate by more than 20%.

Stability

The short-term and long-term stability was determined by analyzing QC samples after keeping at room temperature for 24 h and at -20°C for 14 days, respectively. The auto-injector stability was determined by analyzing QC samples after keeping at 4°C in an auto-injector for 24 h. The freeze and thaw stability was determined by analyzing QC samples after subjecting to 5 freeze-thaw cycles. All stability data were expressed as the percentage of accuracy over nominal concentrations.

Results and Discussion

Mass spectrometry

Figure 1 shows the product ion mass spectra obtained for the protonated riboflavin and \(^{13}\text{C}_4,^{15}\text{N}_2\)-riboflavin (IS). In the full MS2 scan spectrum, the most abundant ions of

![Figure 1. Product ion spectra of the protonated (a) riboflavin and (b) \(^{13}\text{C}_4,^{15}\text{N}_2\)-riboflavin (IS).](image-url)
riboflavin and IS were [M+H]+ at 377.1 and 383.1, respectively. The most prominent fragment ions of the protonated riboflavin and IS were at m/z 242.9 and 249.0, respectively (Figure 1).

All mass spectrometric parameters were optimized for the selectivity and intensity of the protonated ions and its respective product ion. Subsequently, the MRM transitions of m/z 377.1→243.1 for riboflavin and m/z 383.1→248.8 for IS were selected and monitored.

Sample preparation and chromatography

A simple protein precipitation method using acetonitrile was applied to extract riboflavin from the plasma. Protein precipitation is known to have the advantages of simple, rapid, and applicable to high-throughput screening compared to other sample pretreatment methods such as liquid-liquid extraction and solid-phase extraction methods.13,14 However, the efficiency of the simple protein precipitation method to remove endogenous interferences may be lower than those of the other extraction methods.

Due to the low molecular weight and high polarity, riboflavin was co-eluted with polar endogenous interferences in reverse-phase liquid chromatography, leading to ion suppression or low resolution. To separate riboflavin from endogenous substances and to maintain sufficient column retention, hydrophilic interaction liquid chromatography (HILIC) was utilized. The mobile phase composition consisted of 0.1% (v/v) aqueous formic acid with 10 mM ammonium formate and acetonitrile with 0.1% (v/v) formic acid. The sample was separated by using Atlantis HILIC Silica column (150 × 2.1 mm, i.d., 3 μm, Waters, Milford, MA, USA). Since riboflavin is an endogenous compound, 4% bovine serum albumin in phosphate-buffered saline was used as a surrogate matrix to prepare the calibration curve.

Table 2. Equivalency between surrogate matrix and real dog plasma, and process efficiency in surrogate matrix (n = 3, mean ± SD).

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Equivalency (%)</th>
<th>Process efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>92.2 ± 7.8</td>
<td>91.3 ± 1.5</td>
</tr>
<tr>
<td>200</td>
<td>104.7 ± 4.6</td>
<td>89.3 ± 1.3</td>
</tr>
<tr>
<td>400</td>
<td>104.4 ± 4.8</td>
<td>94.0 ± 3.9</td>
</tr>
</tbody>
</table>

MRM chromatograms of riboflavin and IS obtained by extraction of the blank surrogate matrix, surrogate matrix spiked with riboflavin at LLOQ concentration (5 ng/mL), and the dog plasma obtained 4 h after oral administration of the gastroretentive system containing 20 mg of riboflavin are shown Figure 2. Both riboflavin and IS were eluted at 2.8 min, and the total run time was 15 min.

Equivalency and process efficiency

To compare the matrix effect between surrogate matrix and dog plasma, an equivalency test was performed by using low, medium, and high QC samples and the results are shown in Table 2. The average equivalency was 100.4 ± 7.8%, indicating that the matrix effect of the surrogate matrix and dog plasma were comparable.

To evaluate the extraction recovery and matrix effect, the process efficiency of riboflavin was examined at low, medium, and high QC samples in the surrogate matrix (Table 2). Since no extraction step was involved in the sample preparation procedure, the process efficiency directly reflected the matrix effect. The average efficiency was 93.1 ± 3.6%, indicating that the matrix effect was acceptable.

Specificity, sensitivity, and linearity

To examine the impact of endogenous and exogenous substances in the surrogate matrix on analysis, endogenous or exogenous interference tests were conducted. The interferences of matrix and IS on riboflavin were 2.4 ± 1.4% and 1.5 ± 0.2%, respectively, indicating that the impact of endogenous or exogenous interferences on the analysis of riboflavin was minimal.

The calibration range was from 5 to 500 ng/mL. A high linearity was achieved for the calibration curves (r² > 0.9999). The S/N ratio of LLOQ sample was 85.2, indicating that sufficient sensitivity was achieved.

Precision and accuracy

The intra- and inter-day accuracy and precision were determined by assaying LLOQ, low, medium, and high QC samples on the same day (intra-day) and three consecutive days (inter-day). Intra-day and inter-day accuracy and precision data are shown in Table 3. The intra- and inter-day accuracy were 103.5 ± 3.0% and 104.2 ± 3.9%, respectively. The intra- and inter-day precision were within 5.1% and 4.2%, respectively. The obtained intra- and inter-
day accuracy and precision were all within the acceptable ranges suggested by the FDA.  

**Stability**

All stability tests were conducted by using low, medium, and high QC samples. Results of the short-term stability (24 h at 20°C), long-term stability (14 days at -20°C), auto-sampler stability (24 h at 4°C), and freeze-thaw stability (five cycles) in dog plasma are summarized in Table 4. The overall mean percentages of the calculated vs. nominal concentrations were 104.9 ± 4.5% for short-term stability, 103.9 ± 1.9% for long-term stability, 104.4 ± 3.3% for auto-sampler stability, and 106.4 ± 3.7% for freeze-thaw stability. No significant deviations were observed compared to the nominal concentrations, suggesting that riboflavin was stable under all tested conditions.

**Conclusions**

A simple and robust LC-MS/MS method for the determination of riboflavin in Beagle dog plasma was developed. The present method utilized simple protein precipitation with acetonitrile and a stable isotope-labeled riboflavin, $^{13}$C$_4$-$^{15}$N$_2$-riboflavin was used as an internal standard. The developed LC-MS/MS method may provide a useful analytical tool for the in vivo evaluation of new formulations or pharmacokinetic studies of riboflavin.

**Acknowledgments**

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**References**