Quantitative Assessment of the Absolute Purity of Thiopeptcin Reference Standard by $^1$H-NMR

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Quantitative nuclear magnetic resonance (qNMR) has emerged as an easy, rapid and reproducible method for various pharmaceuticals. In the current study, a general qNMR approach for calibrating the purity of the thiopeptcin reference standard (also known as nocathiacin I) was developed using sulfadoxine as an internal standard. Experimental conditions, such as the relaxation delay time and number of scans, were systematically optimized, and the method was validated with different analytical parameters, including selectivity, stability, linearity, precision and robustness. To examine the reliability and feasibility of the present qNMR method, there was no significant difference in the quantification of this complex cyclic peptide compared to the mass balance method. The present study further exemplified that qNMR is a reliable and valuable approach for the assessing of absolute purity of small-molecule pharmaceuticals, which provides a useful tool for drug discovery and development.

**Keywords** Quantitative nuclear magnetic resonance, $^1$H, thiopeptcin, reference standard, method validation, the mass balance method

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**Introduction**

Recently, quantitative nuclear magnetic resonance (qNMR) is a useful instrument to quantify analytes for both academia and industry. According to the definition of CCQM (Comité Consultatif pour la Quantité de Matière), NMR spectroscopy is a primary ratio approach of measurements based on the fact that the response of a NMR signal is proportional to the number of nuclei contributing to the signal.1 Quantification using $^1$H-NMR is widely applied in the quantitative analysis of drugs, excipients, peptides, beverage components, and natural products extracts due to the fact that it has high sensitivity and $^1$H nuclei is widely found in organic molecules.2–6 It can also be used to determine the concentrations of standard solutions in the recent study.7 The main benefit of qNMR is that it can be used in the quantitative assessment of the purity of compounds without the use of some corresponding reference standard. The other advantages—such as a short analysis time, nondestruction, and simple sample preparation—make $^1$H-qNMR a more satisfactory approach for the quantification.

Currently, multidrug resistance has become a main menace to public health worldwide. Thiopeptcin (nocathiacin I, Fig. 1a), a cyclic thiazolyl peptide antibiotic, was separated from the fermentation broth of *Nocardi*a sp. ATCC 202099.8 It exhibits a potent antibacterial activity against a variety of clinically vital multidrug-resistant pathogens, including vancomycin-resistant *Enterococcus* (VRE), methicillin-resistant *Staphylococcus aureus* (MASA), fully penicillin-resistant *Streptococcus pneumonia* (PRSP), and multidrug-resistant *Enterococcus faecium* (MREF).9–11 Thiopeptcin may become a promising candidate to combat bacterial infections due to its very strong activity against various Gram-positive bacterial pathogens.12,13 In addition, thiopeptcin shows superior efficacy in vivo in a systemic *S. aureus* infection mouse model. The mode of action of thiopeptcin and thiazolyl peptide antibiotics is similar. The bacterial growth is prohibited by selectively restraining protein synthesis through interactions with L11 protein and 23S rRNA of the 50S ribosome subunit.14

Reference standards are one important part of the quality-control system of drugs. Their use will be applied throughout the whole drug life cycle. There is no reference substance on sale since thiopeptcin is a kind of first-in-class new drug. Due to the lack of a titration site in its chemical structure, the content of thiopeptcin cannot be determined by volumetric analysis. In our research, we developed a new approach for the quantification...
of the thiopeptcin reference standard using qNMR. Sulfadoxine was used as the internal standard (IS, Fig. 1b). We systematically optimized the experimental conditions containing the relaxation delay time (d1) and the number of scans (ns). We conducted method validation including repeatability and intermediate precision, linearity, robustness, and stability. The developed 1H-qNMR technology was proved to be reliable and feasible by the mass-balance approach. The validation results indicate that 1H-NMR analysis provides plausible purity determination results with a short experiment time, and it is also a supplement procedure and verification of the mass balance approach.15

**Experimental**

**Materials and reagents**

The thiopeptcin reference standard was provided by Nanjing Biotica Pharmaceutical Company. A sulfadoxine reference standard (510119-201501, 99.2%) was obtained from National Institutes for Food and Drug Control (Beijing, China); DMSO-d$_6$ and D$_2$O were bought from Cambridge Isotope Laboratories. The water in the experiment was ultrapure, purified to 18 MΩ cm using a PL5242 Purelab Classic UV (PALL Co., Ltd., USA). All chemicals were of analytical or HPLC grade throughout the experiment.

**Apparatus**

The 1H-NMR experiments were carried out on a Bruker Avance spectrometer operating at 500 MHz with a 5-mm dual-core probe. The XPR10 analytical balance (Mettler Toledo) and the BS 21S analytical balance (Sartorius) were utilized for weighing measurements. High-performance liquid-chromatography (HPLC) experiments were carried out with a Shimadzu LC-20A HPLC system and PHS-25 pH meter was used to measure pH of mobile phase. Gas chromatograph (GC) experiments were performed with an Agilent 6890N GC. The water content was measured by a Mettler Toledo volumetric Karl Fischer titrator.

**Experiment conditions**

In this experiment, the spectral width was set to 13.8 ppm with the center frequency (O1P) at 3.7 ppm. All of the chemical shifts were referenced to the TMS resonance at 0 ppm. Throughout the experiment, the temperature of the NMR probe was 303 K. A relaxation delay time of 20 s was chosen to ensure a full longitudinal relaxation time ($T_1$); the number of scans of 32 were used to ensure the repeatability of the experimental results, and the pulse angle of 30° was adopted to provide a stable experimental condition. Manual shimming was obtained to get a uniform magnetic field, and TOPSPIN 2.1 software with a manual baseline, phase correction and integration was utilized to process data.

**Analysis of reference standard**

For the 1H-NMR experiment, we weighed 46.0 mg of the thiopeptcin reference standard and 10.0 mg of sulfadoxine accurately, and transferred it to EP tubes. After, 1 mL DMSO-d$_6$ and 0.2 mL D$_2$O was added. The solution was mixed completely, and a 0.5-mL portion was transferred to 5-mm NMR tubes. The experiment was carried out under the above conditions, and the results were figured based on the following:16

$$P_X = \frac{I_X}{I_{std}} \frac{N_X}{N_{std}} \frac{M_X}{m_{std}} \frac{m_{std}}{m_X}$$

where $I_X$ and $I_{std}$ are the areas of quantitative signals of thiopeptcin and IS; $N_X$ and $N_{std}$ are the number of protons of thiopeptcin and IS; $M_X$ and $M_{std}$ are the molar mass of thiopeptcin and IS; $m_X$ and $m_{std}$ are the gravimetric weight of thiopeptcin and IS; and $P_X$ and $P_{std}$ are the purity of thiopeptcin and IS, respectively.

**Method validation**

The 1H-NMR method was verified according to the USP 38 guide to general chapters <761> Nuclear Magnetic Resonance Spectroscopy (Analytical Procedure Validation), which includes the stability, robustness, linearity and range, repeatability and intermediate precision.17

**Results and Discussion**

**Choice of appropriate IS, solvent and quantitative signals**

The choice of a proper IS is one of the most significant parts in the qNMR experiment. Also a suitable IS should have some criteria: high purity and good solubility in the chosen solvent. It had better have no reaction with the target substance, and have sharp and isolated peaks in the spectra.18 The single peak of sulfadoxine appears at 6.63 and 8.08 ppm, and its $M_r$ is 310.33 g mol$^{-1}$. It also exhibits have great stability and solubility in the chosen solution. These physico-chemical parameters fulfill the above standard, and hence sulfadoxine is a ideal IS in this method.

In the 1H-NMR experiment, the target drug when completely dissolved in the chosen solvent is one of the most vital principles. Both thiopeptcin and sulfadoxine are soluble in DMSO. But considering that the qNMR peaks of thiopeptcin are too crowded to select isolated peaks for quantitative analysis, we chose a
deuterated solvent to remove exchangeable proton of thiopeptcin, in order to make quantitative peaks isolated. This can ensure precise measurements (Fig. 2). Seeing that thiopeptcin is slightly soluble in water, trifluoroacetic acid (TFA) was added into the mixture to increase the solubility of thiopeptcin. In our pre-experiment, the solvent 1101 μL DMSO-\textit{d}$_6$ + D$_2$O + TFA-\textit{d}$_6$ (800:300:1, v/v/v) was chosen. However, thiopeptcin is not stable in the above-mentioned solution because of the acid condition. After several trials, 1 mL DMSO-\textit{d}$_6$ and 0.2 mL D$_2$O were chosen as desirable solvents for this experiment.

It is reported that the quantitative signals had better having the same chemical environment, and there are basically no sharp and separated signals below 6.0 ppm in the NMR spectra (Fig. 3). The single peaks of thiopeptcin appearing at 7.83, 8.37, and 8.52 ppm were chosen as quantitative signals. Meanwhile, the quantitative signals of sulfadoxine are located at 6.63 and 8.08 ppm. The $^1$H-NMR spectra of thiopeptcin, sulfadoxine (IS), and their mixture are shown in Fig. 4, their quantitative signals are labeled as A, B, C, a and b, which are completely separated in the spectra, illustrating the lack of interference between the two compounds, and indicating the great specificity of this approach.

**Determination of NMR parameters**

In our experiment, we systematically optimized three NMR parameters, including the relaxation delay time, pulse angle and the number of scans.

The relaxation delay time ($d_1$) is an acquisition parameter describing the speed of the proton from the activated state back to ground state. The $d_1$ should be set at least 5-times of $T_1$ to ensure complete relaxation of the corresponding protons. Therefore, $d_1$ is a vital parameter that affects the sensitivity. Consequently, in this part the absolute integral area was examined by changing $d_1$ of five duplicates ($d_1$ = 2, 5, 10, 20 and 40 s). Figure 5 and Table 1 show the influence of various $d_1$ on the absolute integral area of selected signals of the target compound. However, the absolute integral area did not change...
significantly from 20 to 40 s, showing that $d_1 = 20$ s is the saturated parameter for the absolute integral area. Therefore, all tests in this study were selected at $d_1 = 20$ s to enhance the accuracy and reduce the experiment time simultaneously.

A protocol that can act as a standard operation procedure about the widespread spectrometer parameters for qNMR was presented by Malz and Jancke in 2005. A pulse angle at $30^\circ > \theta$ was chosen in this experiment, so that a better $S/N$ and a much more stable experiment condition can be obtained in a short experiment time. Therefore, we also tested the effect of the number of scans on the absolute integral area of the quantitative signals by changing the number of scans of five duplicates of 46.0 mg of the thiopeptcin reference standard dissolved in chosen solutions at 2, 4, 8, 16, 32 and 64. Figure 5 and Table 1 show the influence of different numbers of scans on the absolute integral area of the selected signals of the target compound. Also the relative integral area was calculated, showing that it remains steady when $n_s$ is 32 or 64 times. Thus, in general, $n_s$ at 32 times was selected in order to enhance the accuracy and to shorten analysis time simultaneously.

Method validation

Repeatability and intermediate precision. The repeatability and intermediate precision were evaluated by analyzing six prepared samples independently with five duplicates in two different days. Two different analysts conducted sample preparations and analyses. The mean content of drug in the percentage assay and relative standard deviation (RSD) are shown in Table 2. The average content of the reference standard was 95.72 and 95.53%, and RSDs were found to be 0.73 and 0.29% for repeatability and intermediate precision analysis. The outcomes clearly indicated great accuracy and precision for the $^1$H-NMR method, which is in a position to assess the absolute content of the target drugs. Linearity. There is a linear relationship between the instrument response and the concentration of the analyte. The linearity of the developed $^1$H-NMR method was verified from 50 to 150% ($n_s = 5$) by preparing five varying concentrated samples. The relevant equation was $y = 0.9467x + 0.0292$ with a correlation coefficient $R^2 = 0.9996$, which was processed using IBM SPSS statistics 22.0 software. In this equation, $y$ was the ratio between the integral area of thiopeptcin and the IS, $I_a$ and $I_b$ are the areas of quantitative signals of thiopeptcin and IS.

![Fig. 6 Linearity curve of thiopeptcin of the molar ratio, $n_a/n_b$, versus the integration area, $I_a/I_b$ (($n_a$ and $n_b$ are the molar concentration of thiopeptcin and IS, $I_a$ and $I_b$ are the areas of quantitative signals of thiopeptcin and IS)).](image-url)
Stability. The stability of thiopeptacin was determined at 2 h intervals (0, 2, 4, 8 and 12 h) by measuring duplicates (n = 5) of the same sample. The results (Table 3) demonstrated that the samples were unaffected by 12 h of storage at room temperature with the IS and solvent. Also, RSD% values were less than 1%.

Robustness. In the analytical procedures, the robustness is a determination of its ability not to be affected by little, but significant, variations.14 The robustness of this method was validated through changing three critical experimental parameters apart: the number of scans (32 ± 8), the probe temperature (303 ± 5 K) and the weight of IS (10.0 ± 1.0 mg).18 The considered parameters and their changes are generalized in Table 4. The outcomes shown that: the different number of scans at 24, 32 and 40 did not affect the content of the target compound; a small variation in the weight of the IS at 9.0, 10.0, and 11.0 mg gave similar results; varying the probe temperature at 298, 303, and 308 K were almost unchanged in the measured content of the compound. Consequently, the robustness of the 1H-NMR method meets the provisions based on these three parameters mentioned above.

Comparison with the mass balance approach

To illustrate the availability of the developed 1H-NMR approach, the outcomes acquired in aforementioned method were compared with the mass-balance approach results. The mass balance approach involves quantifying all impurities (including organic, inorganic, water, and solvents) from 100% under Eq. (2).21

Content % = 100% – impurity % – water % – residual solvents % – ash %

A reverse-phase HPLC (Shimadzu LC-20A) with diode array detector (DAD) was utilized to analyze the impurity of the thiopeptacin reference standard. The seperation was conducted on a Phenomenex C 18 column (250 × 4.6 mm, 5 μm) with gradient elution. The linearity of the DAD detector and the repeatability of the HPLC were also studied before determining of the purity.22 A quantitative calculation was carried out by the area normalization method.

The GC method determined residual solvents in the reference standard. The experiment was quantified by an Agilent 6890N GC equipped with a flame ionization detector. A 30 m × 0.32 mm DB-W AX column (0.25 μm film thickness) was used with temperature programmed run. The quantitative calculation was carried out by the external standard method.

The results of the water content for thiopeptacin were measured by the Karl Fischer titration standard operational procedure. The determinations of sulfated ash were carried out by conventional methods.23 The results showed that the absolute content of the thiopeptacin reference standard was 95.00% by the mass-balance method, whereas it was 94.84% by means of the qNMR method with five duplicates (shown in Table 5). The calculations of the contents of thiopeptacin were almost the same.

Table 3 Stability results of thiopeptacin reference standard under storage conditions (n = 5)

<table>
<thead>
<tr>
<th>Time interval/h</th>
<th>Taken/mg</th>
<th>Found/mg</th>
<th>$I_s$</th>
<th>$I_R$</th>
<th>Assay, %</th>
<th>Mean, %</th>
<th>RSD, %</th>
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<tr>
<td>0</td>
<td>47.77</td>
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<td>394983590</td>
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<td>2</td>
<td>47.77</td>
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$I_s$: The absolute integral area of thiopeptacin. $I_R$: The absolute integral area of IS.

Table 4 Robustness tests of thiopeptacin reference standard

<table>
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<tr>
<th>Parameter</th>
<th>Change</th>
<th>Taken/mg</th>
<th>Found/mg</th>
<th>$I_s$</th>
<th>$I_R$</th>
<th>Assay, %</th>
<th>Mean, %</th>
<th>RSD, %</th>
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<tr>
<td>Number of scans</td>
<td>24</td>
<td>44.16</td>
<td>41.84</td>
<td>318727656</td>
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<td></td>
<td>32</td>
<td>44.16</td>
<td>41.84</td>
<td>424600152</td>
<td>447226016</td>
<td>94.58</td>
<td>94.81</td>
<td>0.12</td>
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<tr>
<td>Probe temperature/K</td>
<td>40</td>
<td>44.16</td>
<td>41.93</td>
<td>529851599</td>
<td>559128286</td>
<td>94.56</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>303</td>
<td>44.75</td>
<td>42.32</td>
<td>422701660</td>
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<td>94.80</td>
<td>0.43</td>
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<td></td>
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<td>42.31</td>
<td>388971062</td>
<td>409152347</td>
<td>94.94</td>
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<tr>
<td>IS/mg</td>
<td>9</td>
<td>40.52</td>
<td>38.45</td>
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<td>418883900</td>
<td>94.88</td>
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<td>10</td>
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$I_s$: The absolute integral area of thiopeptacin. $I_R$: The absolute integral area of IS.

Table 5 Content of thiopeptacin reference standard by 1H-NMR method (n = 3)

<table>
<thead>
<tr>
<th>No.</th>
<th>Taken/mg</th>
<th>Found/mg</th>
<th>$I_s$</th>
<th>$I_R$</th>
<th>Assay, %</th>
<th>Mean, %</th>
<th>RSD, %</th>
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<tr>
<td>1</td>
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<td>47.25</td>
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<td>427819160</td>
<td>420453881</td>
<td>94.72</td>
<td>94.85</td>
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<tr>
<td>3</td>
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<td>42.90</td>
<td>415510645</td>
<td>466489314</td>
<td>94.55</td>
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</table>

$I_s$: The absolute integral area of thiopeptacin. $I_R$: The absolute integral area of IS.
Conclusions

1H-NMR demonstrated the advantages of the selectivity and reproducibility in the quantitative analysis of thiopeptcin. Moreover, other benefits of qNMR contain easy sample preparation, a rather rapid and simple analysis, and detection speed. The qNMR’s calculations of the contents of thiopeptcin were almost the same as those of the mass-balance approach. The developed qNMR approach could be used as a feasible approach for the assay of standard references.

Nuclear magnetic resonance is an appealing platform for conducting quantification studies on complex compounds. However, there are some limits of qNMR about data processing. Although the experiment time is short, it is spent a lot of time to do the data processing. In 2017, Monakhova and Diehl developed and validated a new technique for automated spectra integration and quality control of the acquired results in qNMR. The technique can save time based on the fully automated manner which only needs simple manual improvement or quick check for plausibility. Furthermore, it can increase the accuracy and reproducibility, and be free of systematic and user-specific errors.24,25

Although the qNMR method spends much time to do the data processing, it still uses less time comparing with the mass balance approach. Also, with development of the new technique, qNMR method is still the preferred choice for the absolute purity of innovative drugs reference standards.

Acknowledgements

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References