

# Quantitative proton magnetic resonance determination of N,N-dimethylformamide in one intermediate of the Quimi-Hib vaccine

Raine Garrido Arteaga,<sup>a\*</sup> Felix Cardoso San Jorge,<sup>a</sup>  
María del C. Rodríguez Montero,<sup>a</sup> Violeta Fernández Santana,<sup>a†</sup>  
Vicente Vérez Bencomo<sup>a</sup> and Herman Vélez Castro<sup>a</sup>

Quimi-Hib is a conjugate vaccine against *Haemophilus influenzae* type b (Hib) where the Hib antigen is the only one produced by chemical synthesis. NMR has become the alternative of choice for the identity of intermediates during the chemical synthesis of Hib antigen. We explore a rapid quantitative proton magnetic resonance (qHNMR) assay for the determination of N,N-dimethylformamide (DMF) as a residual in one of the critical intermediates. The proposed assay has been shown to be accurate, precise for intermediate precision conditions (relative standard deviation <3% for spectrometer-to-spectrometer variations), specific (no detected interferences), and rugged (percentage difference <3% for day-to-day and spectrometer-to-spectrometer variations). The quantitative NMR assay can replace the common chromatographic methods for monitoring the DMF contents in one crucial step of the synthetic scheme. Copyright © 2012 John Wiley & Sons, Ltd.

**Keywords:** NMR; proton; quantitative analysis; Hib; vaccine

## Introduction

*Haemophilus influenzae* type b (Hib) is a Gram-negative bacterium that causes meningitis, pneumonia, and otitis media, mainly in children under 4 years old. The active pharmaceutical ingredient (API) of Quimi-Hib vaccine is composed of Hib antigen obtained by chemical synthesis and further conjugated to tetanus toxoid.<sup>[1]</sup> NMR is one of the most widely used techniques in the evaluation of active ingredients and intermediates of high added value.<sup>[2]</sup> In the last decade, the application of quantitative proton magnetic resonance (qHNMR) had an increasing impact on the pharmaceutical industry. The qHNMR method involves proton NMR experiments with several modified parameters in order to obtain spectra with quantifiable signals (99.9% recovery of equilibrium magnetization). These parameters depend essentially on the relaxation times of nuclei to be evaluated for the analyte and the reference.<sup>[3]</sup> There are theoretical values for the parameters that yield the best qHNMR spectra. These assays usually take hours depending on the analyte and reference quantities in the sample. However, the parameters can be optimized experimentally, decreasing the delay of the assay. This paper discusses a method of qHNMR developed for the control of N,N-dimethylformamide (DMF) in 5-O-Allyl-1-O-(2,5-di-O-benzyl-β-D-ribofuranosyl)-2,3,4-tri-O-benzyl-D-ribitol (compound 1) that represents one critical intermediate of the synthesis scheme of Hib antigen<sup>[4]</sup> (Fig. 1).

## Materials and Methods

### Sample preparation

The sample of compound 1 was supplied by the Quality Department of the Center for Genetic Engineer and Biotechnology. Seven

deuterated chloroform stock solutions were prepared with 1.7 mg ml<sup>-1</sup> tert-butanol (t-BuOH) as internal reference for qHNMR, 0.2 mg ml<sup>-1</sup> TMS, and different quantities of DMF [7.5 mg ml<sup>-1</sup> (A), 8.3 mg ml<sup>-1</sup> (B), 10.2 mg ml<sup>-1</sup> (C), 11.5 mg ml<sup>-1</sup> (D), 12.8 mg ml<sup>-1</sup> (E), 14.2 mg ml<sup>-1</sup> (F), and 15.5 mg ml<sup>-1</sup> (G)] using 5 ml (±0.5%) volumetric flasks. Three sets of samples were prepared by dissolving 10 mg of compound 1 in 0.6 ml of each of the above-mentioned stock solutions. All reagents and solvents were of spectroscopic quality.

### Instrument setting

NMR analyses were carried out on two Bruker Avance DPX 250 instruments operating at 250.13 MHz. The main spectrometer (A) operates with a 5-mm quadrupole nuclei probe (<sup>1</sup>H, <sup>13</sup>C, <sup>31</sup>P, and <sup>19</sup>F), whereas the second spectrometer (B) operates with a 5-mm <sup>1</sup>H and <sup>13</sup>C dual probe, both at a temperature of 27 °C. The inversion–recovery experiment was run with a list of 26

\* Correspondence to: Raine Garrido Arteaga, Center of Biomolecular Chemistry, 200 Street and 21 Ave., Atabey, Playa, La Habana, Cuba 11600. E-mail: raine.garrido@cqb.cu

† In memory of Dr Violeta Fernández Santana, who passed away on 20 November 2011.

a Center of Biomolecular Chemistry, 200 Street and 21 Ave. Atabey, Playa, La Habana, Cuba, 11600

**Abbreviations used:** Hib, *Haemophilus influenzae*, type b; API, active pharmaceutical ingredient; qHNMR, quantitative proton magnetic resonance; DMF, N,N-dimethylformamide; t-BuOH, tert-butanol; QNP, quadrupole nuclei probe; ICH, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; RSD%, relative standard deviation.

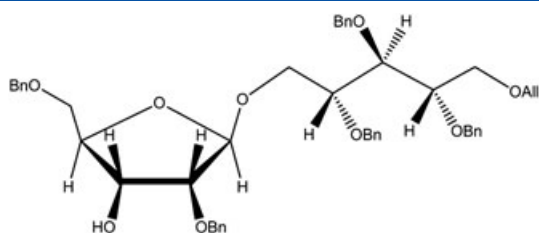


Figure 1. Structure of compound 1.

values of delays between proton pulses for the relaxation time ( $T_1$ ) calculation and 60 s of relaxation delay.<sup>[5]</sup>

The qHNMR-optimized experiment was set with a spectral width of 2997.6 Hz (12 ppm), 256 scans after 4 steady state scans, an Ernst angle of  $56^\circ$  for the excitation pulse, decoupling of  $^{13}\text{C}$  nuclei through GARP sequence, 7 s of relaxation delay, and a recycled time (12 s) ensuring more than five times the longest  $T_1$  of interest. The TMS signal was set at  $\delta$  0.00 ppm to calibrate the spectra. Apodization process was carried out by an exponential function (LB 0.3 Hz). To guarantee the signal integration process, a third-order polynomial fit and manual phase correction were required. The spectral data were acquired and transformed under TOPSPIN 1.3 suite.

The signal-to-noise ratio ( $s/n$ ) was calculated with the Sinocal microprogram included in the TOPSPIN 1.3 suite.

### Assay evaluation and statistical analysis

The quantitative proton NMR method was evaluated according to Schofield and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines.<sup>[6,7]</sup> The assessments were carried out with respect to the following performance parameters: accuracy, repeatability, intermediate precision for inter-apparatus variations, linearity, specificity, and ruggedness to changes of test day and spectrometer employed.

## Results and Discussion

### qHNMR assay: specificity and $T_1$ measurements

The specificity assay was carried out to check the specific contribution of the analyzed components (DMF and t-BuOH). The evaluation focused on the absence of interference signals from the intermediate and any other component of the sample in the evaluated regions. In the proton NMR spectrum, the amide proton of DMF (H-DMF) gave rise to a singlet at  $\delta$  8.01 ppm. In addition, the typical signals of compound 1 appeared between  $\delta$  7.50 and 3.40 ppm. The singlet at  $\delta$  1.27 ppm (H-reference) belonged to the methyl signals of t-BuOH.<sup>[8]</sup> No interferences were found for the evaluated regions between  $\delta$  7.91–8.11 ppm and  $\delta$  1.17–1.37 ppm (I-1 and I-2, respectively) (Fig. 2).

In order to measure the spin-lattice relaxation rate of the evaluated protons (H-DMF and H-reference), the inversion-recovery experiment was performed. The  $T_1$  values measured were 1.75 and 1.90 s for DMF and t-BuOH, respectively. The optimized qHNMR assay finally fitted with an Ernst angle of  $56^\circ$ , a relaxation delay of 7 s, and a total recycled delay of 12 s, five times the  $T_1$  value. In addition, 256 scans were collected to ensure a good  $s/n$  for the DMF evaluation.<sup>[9]</sup> In theory, any other reagent with a  $T_1$  value lower than 1.90 s and without interferences in the spectrum can also be evaluated.

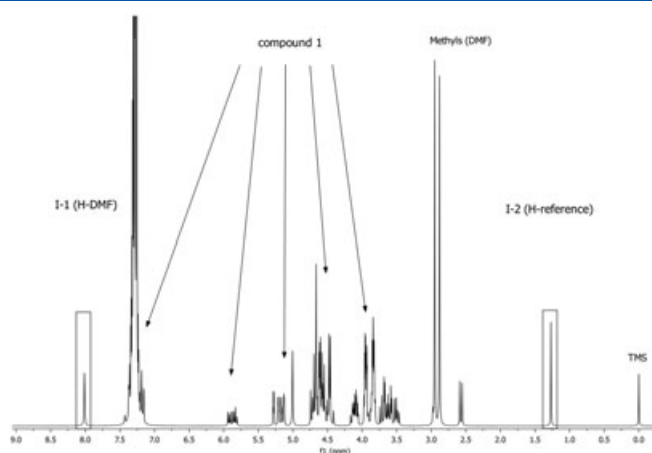


Figure 2. qHNMR-optimized spectrum of compound 1 with DMF.

### Range for qHNMR assay

As a strategy for monitoring potential residuals, all the reagents and solvents employed in the synthesis scheme of the API were evaluated step by step at different stages. To meet the regulatory requirements, an acceptance criterion equivalent to  $11 \text{ mg mL}^{-1}$  of DMF in the sample was priorly established. In order to ensure results with adequate linearity, accuracy, and precision in the vicinity of acceptance criterion, we selected a range for statistical evaluation between 7.5 and  $15.5 \text{ mg mL}^{-1}$ .<sup>[7]</sup> Three sets of seven samples containing DMF, t-BuOH, TMS, and compound 1 in deuterated chloroform were prepared.

### Calculations from the spectrum

Quantitative proton NMR analysis is based on the peak integration of amide proton from DMF and methyl protons from t-BuOH at chemical shift regions between  $\delta$  7.91–8.11 ppm and  $\delta$  1.17–1.37 ppm, respectively. Equation (1) shows the calculation according to the absolute method from the integrated proton peaks.<sup>[10]</sup>

$$\text{DMF\%} = \frac{I_1}{I_2} \times \frac{M_{\text{DMF}}}{M_{\text{t-BuOH}}} \times \frac{m_{\text{t-BuOH}}}{m_{\text{sample}}} \times 9 \times P_{\text{t-BuOH}} \quad (1)$$

The constant 9 in Eqn (1) is the number of methyl protons in t-BuOH,  $M$  is the molar mass of DMF and the reference (t-BuOH),  $m$  is the mass of the reference and the total sample (DMF contents of the aliquot and compound 1 of each sample),  $P$  is the purity of the reference reported by the supplier, and  $I_1$  and  $I_2$  are the integral values in the spectrum (Fig. 2). Table 1 shows the results obtained by the assay.

Table 1. Theoretical and practical results in the evaluation of DMF content

Theoretical	Measured (set 1)	Measured (set 2)	Measured (set 3)
%DMF (%)	%DMF (%)	%DMF (%)	%DMF (%)
31.44	30.59	31.02	31.02
34.85	35.39	34.64	35.02
37.93	39.27	37.93	39.60
40.73	40.87	38.94	39.92
43.29	43.57	42.68	43.85
45.63	45.45	46.22	44.95
47.78	47.76	47.27	48.03

### Accuracy and linearity

Accuracy, per ICH's advice, should be reported as percent recovery by the assay of the known added amounts of analyte in the sample. The accuracy assessment was carried out with 21 determinations over one set of seven samples with known amounts of DMF (three replicates per sample). The mean for percent recovery by the proposed assay ( $\text{DMF\%}_{\text{recovery}}$ ) and the confidence intervals (95%) were calculated through hypothesis testing.

$$\text{DMF\%}_{\text{recovery}} = \frac{\text{DMF\%}_{\text{measured}}}{\text{DMF\%}_{\text{theoretical}}} \times 100 \quad (2)$$

$\text{DMF\%}_{\text{measured}}$  is the estimated DMF% value from the NMR spectral analysis using Eqn (1), and  $\text{DMF\%}_{\text{theoretical}}$  is the expected value for each sample. The mean value for  $\text{DMF\%}_{\text{recovery}}$  was 99.87%, and the confidence intervals for 95% were 98.97% to 100.77%.<sup>[7]</sup>

The linearity for DMF% was analyzed using three set of samples, each similar to the employed set for accuracy analysis. Xu *et al.* have employed proportional bias as a linearity criterion (proportional bias  $\leq 10\%$ ).<sup>[11]</sup> The proportional bias was calculated according to Eqn (3).<sup>[6]</sup>

$$\text{prop. bias} = (2^{|s-1|} - 1) * 100 \quad (3)$$

where  $s$  is the estimated slope for the linear regression analysis of the  $\text{DMF\%}_{\text{measured}}$  result versus  $\text{DMF\%}_{\text{theoretical}}$ . The slope value of the linear regression was 0.9989, and the resultant proportional bias was 0.08% (Fig. 3).

### Intermediate precision and repeatability

For the repeatability assessment, a piece of data from 21 determinations (similar to accuracy assay) was processed. The generated results were used to calculate the relative standard deviation

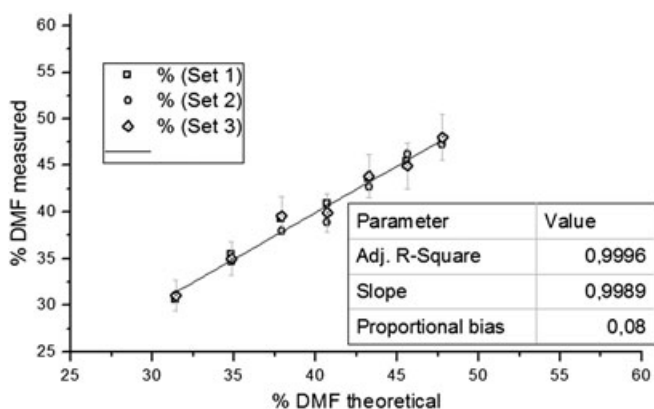


Figure 3. Linear regression plot of linearity assessment.

(RSD%) In addition, 14 determinations (two replicates) were carried out in spectrometer (B) as variation to calculate the intermediate precision. The resultant means of  $\text{DMF\%}_{\text{recovery}}$  were 99.87% for repeatability and 99.45% for intermediate precision. The RSD% values were 2.03% and 2.49% for repeatability and intermediate precision, respectively<sup>[7]</sup> (Table 2).

### Quantification limit

The quantification limit (QL) is the lowest test result of a sample that can be quantitatively determined with suitable precision, accuracy, and linearity. Many instrumental methods employ Eqn (4) in determining QL. Soininen *et al.* assessed the QL of an NMR method for impurity quantification by employing constrained total-line-shape fitting.<sup>[12]</sup> However, ICH advises as alternative assessment for those assays where the  $s/n$  can be evaluated that it must be better than 10:1.<sup>[7]</sup> For evaluating the assay under the  $s/n$  criterion, several dilutions of the analyte must be acquired until a ratio close to the ICH recommendation is achieved. Diehl *et al.* proposed an  $s/n$  of at least 250:1 for a precision better than 1% for qHNMR methods.<sup>[13]</sup>

$$\text{QL} = 10 \times \sigma / s \quad (4)$$

where  $\sigma$  is the standard deviation of the response and  $s$  is the slope of the calibration curve.

In order to ensure that the evaluated range was above the QL, we focus on evaluating the  $s/n$  employing the H-DMF peak at 8.01 ppm for the DMF less concentrated sample (A) and a noise range from 11 to 9 ppm in the spectrum. Due to the fact that our lowest DMF concentration ( $7.5 \text{ mg mL}^{-1}$ ) was 31 times higher than the ICH recommendation, it was not necessary to calculate the actual QL for the method.

### Ruggedness

Ruggedness was evaluated using pairwise averaged percentage differences of measurements by two different spectrometers (A and B) on three different days with three different samples of intermediate contaminated with different quantities of DMF. The results for the assay were expressed as averaged inter-apparatus percentage difference and averaged inter-day percentage difference. The averaged percentage difference was calculated according to Eqn (5).

$$\% \text{Difference} = (e^{|\bar{x}-\bar{y}|} - 1) * 100 \quad (5)$$

where  $x$  is the within-sample mean of the logarithm-transformed result for spectrometer A (or day 1) and  $y$  is the analog result for spectrometer B (or day 2).

Table 2. Summary of statistical analysis

Parameter assessed	Acceptance criteria	Result
Linearity	Proportional bias $\leq 10\%$	0.08% bias
Accuracy	The average percentage recovery versus expected values: $\text{DMF\%}_{\text{recovery}} 100\% \pm 20\%$	99.87% recovery
Range	Determined according to quality specification for DMF	$7.5\text{--}15.5 \text{ mg mL}^{-1}$
Precision	Repeatability: $\text{RSD\%} \leq 5\%$	2.03% RSD
Precision	Intermediate precision: $\text{RSD\%} \leq 10\%$	2.49% RSD
Quantification limit	Will be determined according to adequate accuracy, precision, and linearity	$7.5 \text{ mg mL}^{-1}$
Ruggedness	Inter-day percentage difference: $\% \text{Difference} \leq 10\%$ on average	1.14% difference
Ruggedness	Inter-apparatus percentage difference: $\% \text{Difference} \leq 10\%$ on average	0.21% difference

The maximum inter-day percentage difference was 1.14%, and the major percentage difference value from spectrometer A to spectrometer B was 0.21%. The acceptance criterion was similar to that employed by Xu *et al.*<sup>[11]</sup> (Table 2).

## Conclusions

The qHNMR-optimized assay provides a valuable procedure to evaluate DMF in compound 1, a critical intermediate of the Quimi-Hib vaccine. Further studies can successfully extend the results to monitor other impurities or broader ranges. The statistical assessment for the studied range demonstrated good linearity, accuracy, precision (under intermediate precision conditions), and acceptable ruggedness. It is tolerable to changes in instruments and days of analysis.

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