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Improving the efficiency of quantitative ^{1}H NMR: An innovative external standard–internal reference approach

Yande Huang*, Bao-Ning Su[1], Qingmei Ye, Venkatapuram A. Palaniswamy[2], Mark S. Bolgar, Thomas V. Raglione

Analytical and Bioanalytical Development, Bristol-Myers Squibb, New Brunswick, NJ 08903-0191, USA

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ABSTRACT

The classical internal standard quantitative NMR (qNMR) method determines the purity of an analyte by the determination of a solution containing the analyte and a standard. Therefore, the standard must meet the requirements of chemical compatibility and lack of resonance interference with the analyte as well as a known purity. The identification of such a standard can be time consuming and must be repeated for each analyte. In contrast, the external standard qNMR method utilizes a standard with a known purity to calibrate the NMR instrument. The external standard and the analyte are measured separately, thereby eliminating the matter of chemical compatibility and resonance interference between the standard and the analyte. However, the instrumental factors, including the quality of NMR tubes, must be kept the same. Any deviations will compromise the accuracy of the results. An innovative qNMR method reported herein utilizes an internal reference substance along with an external standard to assume the role of the standard used in the traditional internal standard qNMR method. In this new method, the internal reference substance must only be chemically compatible and be free of resonance-interference with the analyte or external standard whereas the external standard must only be of a known purity. The exact purity or concentration of the internal reference substance is not required as long as the same quantity is added to the external standard and the analyte. The new method reduces the burden of searching for an appropriate standard for each analyte significantly. Therefore the efficiency of the qNMR purity assay increases while the precision of the internal standard method is retained.

1. Introduction

In pharmaceutical industry, most assay methods for the determination of purity (wt%) utilize reference standards to calibrate the detector response. The purity of reference standards must be well established. In the absence of a qualified reference standard determination of purity is usually conducted by the mass balance method, an indirect procedure for assigning purity by determining the wt% of all unrelated species such as residual solvents, inorganic materials, moisture, etc. Although this approach avoids the need for a reference standard to quantify the analyte, a significant limitation is the requirement that every species present in the sample must be quantified. Meeting this requirement is challenging especially during the early stages of development where the quality of the analyte may not be well established and a full suite of analytical methods may not be available. Any overlooked species will directly affect accuracy of the purity assignment.

In contrast, the traditional quantitative ^{1}H NMR (qNMR) method utilizes a structurally distinct reference standard as an internal standard to directly determine the purity of the analyte. Any impurities, either organic or inorganic or moisture, will not affect the purity measurement of the analyte as long as they do not possess any resonance that interferes with the one selected for integration for the analyte and the internal standard. This makes qNMR an excellent orthogonal quantitative method to ensure the accuracy of the purity assignment of the analyte because appropriate qNMR internal standards are available from United States Pharmacopeial Convention (USP) or National Institute of Standards and Technology (NIST).

The accuracy and precision of the internal standard qNMR method have been reported to be comparable to those of the well-established and widely used analytical HPLC method [1–3]. In these studies each analyte and the internal standard are present in the same solution. The instrumental factors which are collectively reflected in the spectrometer constant [4], are identical for

* Corresponding author at: Analytical & Bioanalytical Development, Bristol Myers-Squibb, One Squibb Drive, New Brunswick, NJ 08903, USA.
Tel.: +1 732 227 7405; fax: +1 732 227 3934.
E-mail address: yande.huang@bms.com (Y. Huang).
[1] Present address: WuXi AppTec Co., Ltd., 288 Fute Zhong Road, Waigaoqiao Free Trade Zone, Shanghai 200131, China.
[2] Present address: Novatia, LLC, 11 Deer Park Drive, Suite 202, Monmouth Junction, NJ 08852, USA.

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the analyte and standard. Therefore, the quantitative relationship between the analyte and the internal standard is established, as shown in Eq. (1).

$$\frac{I_1}{I_2} = \frac{n_1}{n_2} \times \frac{W_1}{W_2} \times \frac{M_2}{M_1} \times \frac{P_1}{P_2} \quad (1)$$

where, I, integral of selected resonances of the analyte (**1**) and internal standard (**2**); n, number of protons in the respective functional groups related to the resonances of the analyte (**1**) and internal standard (**2**); W, weight of the analyte (**1**) and internal standard (**2**); P, purity (wt%) of the analyte (**1**) and internal standard (**2**); and M, molecular weight of the analyte (**1**) and internal standard (**2**).

As the internal standard and the analyte co-exist in the same solution, there are certain restrictions in the selection of a suitable qNMR internal standard. The internal standard must be compatible with the analyte of interest, i.e. no overlap of the key resonances used for integration to satisfy the resonance specificity and no chemical reaction between the internal standard and the analyte during NMR sample preparation and data acquisition. In addition, the internal standard must have a known purity and good solubility in the NMR solvent to be used. Therefore, finding a suitable internal standard can be a time-consuming undertaking in internal standard qNMR method development.

Besides the internal standard qNMR method there is an alternative qNMR method that uses an external standard to calibrate the NMR instrument for the subsequent measurements of the concentrations of analytes, such as proteins [5] and natural products [6]. In the external standard qNMR method, the same volume of analyte and external standard solutions were placed in separate NMR tubes of identical precision for NMR data acquisitions. The NMR probe was tuned and matched, shimming was optimized for each measurement, and the 360° pulse length was precisely determined for the external standard and the analyte, respectively. The reported precision and accuracy were comparable to those reported for internal standard methods. Another similar approach was the so-called ERETIC method [7,8], using a synthesized electronic reference signal which was calibrated by an external standard. In these external standard qNMR methods, an external standard could be easily selected without any concerns about its compatibility with the analytes. However, the instrumental factors need to be kept the same because the spectra of the external standard and the analyte were acquired separately. Any variations generated from differences in the NMR tubes can affect the accuracy of the results.

An innovative qNMR method reported herein uses an external standard with a known purity and an internal reference substance to assume the roles of the internal standard used in the traditional internal standard qNMR method. The external standard is treated as one of the analytes during NMR sample preparation and data acquisitions, while the same amount of internal reference substance is added to each individual preparation of the analyte and external standard. In this case, the internal reference substance only serves as a reference point through which the integrals of the external standard and the analyte are linked together for purity calculation. The exact purity and accurate weighing of the internal reference substance are not required. Therefore, the compatibility issues between the internal reference substance and the external standard or the analyte are readily resolved because more choices are available for internal reference substance without the requirement of knowing its exact purity. Details of the new method are discussed below and illustrated with an example.

2. Conceptual analysis

2.1. Concept

The principle of this new external standard/internal reference substance qNMR method can be illustrated by preparing two typical NMR samples: sample-1 contains an analyte (**1**) and an appropriate internal reference substance (**2**), while sample-2 contains the same internal reference substance (**2**) and an external standard (**3**). The relationship between analyte (**1**) and internal reference substance (**2**) in sample-1 is established by Eq. (1). Similarly, internal reference substance (**2**) and external standard (**3**) in sample-2 can be described in Eq. (2).

$$\frac{I_3}{I_2} = \frac{n_3}{n_2} \times \frac{W_3}{W_2} \times \frac{M_2}{M_3} \times \frac{P_3}{P_2} \quad (2)$$

If the weight of internal reference substance (W_2) is kept the same in sample-1 and sample-2 during NMR sample preparation and its integrals (I_2) of the same resonance in spectrum-1 (sample-1) and spectrum-2 (sample-2) are calibrated to the same value, the relationship between analyte (**1**) and external standard (**3**) can be established in Eq. (3) which is derived from the division of Eq. (1) by Eq. (2). Note that all terms related to the internal reference substance (**2**) are canceled out.

$$\frac{I_1}{I_3} = \frac{n_1}{n_3} \times \frac{W_1}{W_3} \times \frac{M_3}{M_1} \times \frac{P_1}{P_3} \quad (3)$$

In this method, the exact purity of the external standard (**3**) rather than that of the internal reference substance (**2**) will be used in the purity calculation of the analyte. The internal reference substance only serves as a reference point in data acquisition and processing and its exact purity and accurate weighing are not required if an equal amount of the internal reference substance solution prepared in a suitable NMR solvent is dispensed into each of the individual samples of the analyte and the external standard. This simplifies the sample preparation and increases the efficiency of the method. This internal reference substance/external standard qNMR method can be easily extended to analyze a group of analytes simultaneously by sharing only one external standard.

As a quantitative method, the data acceptance criteria should be established and verified. In this new method, a second external standard can be readily introduced as a system suitability check. The acceptance criteria of the qNMR method can be set by comparing the measured purity of the second external standard with its established purity. Thus, each qNMR experiment will be comprised of a set of samples including an external standard, a system suitability check standard and all of the analytes of interest. Each sample contains the same amount of a common internal reference substance. Other basic requirements, such as the specificity (or purity) of the selected resonances for integrations must be met for each of the standards and analytes to achieve accurate results. This is common requirement for any qNMR methods.

2.2. General formula for analyte purity calculation

If replicate measurements for each sample are conducted, the purity of the analytes and the system suitability check standard (P_1, wt%) can be calculated using Eq. (4) which is derived from Eq. (3). The weighted integral (I_3/W_3) of the external standard (**3**) is kept intact so that its averaged value from replicate measurements can be used for purity calculation of each of the individual analytes and the system suitability check standard. The relative standard deviation (% RSD) of (I_3/W_3) can be used to measure the precision if three or more replicate samples of external standard are analyzed. For each of the individual analytes and the system suitability check