ADVANTAGES OF QUANTITATIVE NMR FOR THE DETERMINATION OF RELATIVE RESPONSE FACTORS

Quantitative NMR (qNMR) is a technique that is being applied broadly and at an increasing rate in the field of pharmaceutical analysis (1). This white paper highlights the advantages of using qNMR to determine Relative Response Factors (RRFs) for pharmaceutical impurities detectable by HPLC. A single determination of RRFs using qNMR allows for simple and accurate quantitation of impurities which eliminates the need for preparation, qualification, and storage of reference standards. An example is presented here, which demonstrates quantitation of known impurities that have variable responses to UV-VIS detection, thereby providing a more accurate assessment of impurity levels than UV-VIS response alone.

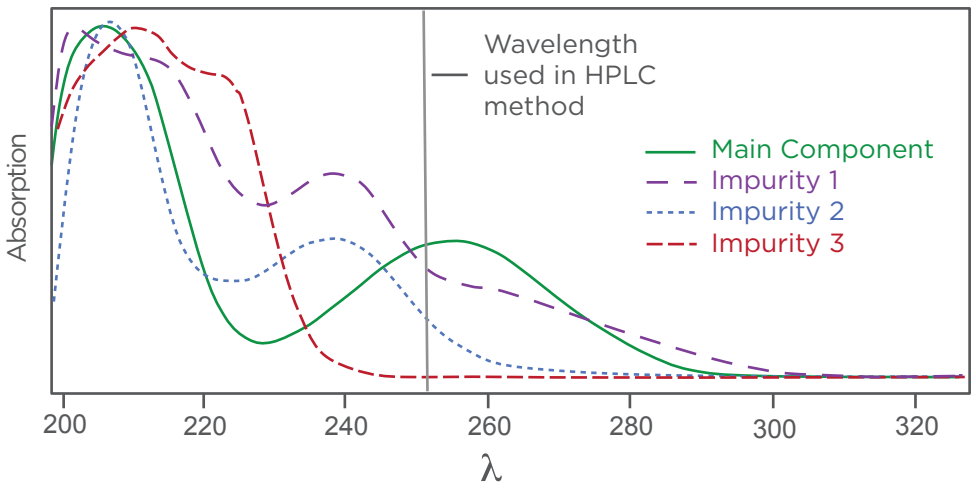


FIGURE 1: Varied UV-Vis absorbances of several species in a sample. Note that at the wavelength of interest, 254 nm, the Main Component shows the highest UV absorption while Impurity 3 shows little to no absorption.

BACKGROUND

Under established ICH guidelines, analytical methods must be developed and validated to ensure that impurities present in Active Pharmaceutical Ingredients (APIs) and formulated drug products are controlled within set specifications agreed upon at the time of product registration and approval. Residual starting materials, intermediates, by-products and/or degradants need to be controlled against established specifications at each manufacturing stage for synthetic APIs. The standard analytical methodology employed for this purpose is high performance liquid chromatography (HPLC) with UV-VIS detection.

A direct area percent measurement of a UV chromatogram at a single wavelength is a quick, useful assessment of purity when comparing one sample to another. However, it lacks specificity for measuring the actual quantity of any given substance [Figure 1].

The true level of a given impurity or degradant is of great importance from a pharmaceutical regulatory viewpoint, since over-reporting an impurity may lead to unnecessary efforts to remove the impurity, while under-reporting the impurity may run the developer afoul of prescribed ICH limits. Commonly, one of two approaches is considered to measure the true level of a specific known impurity in a given sample: 1) assay the sample against a well-characterized standard of known purity, or 2) determine and apply the impurity's response factor relative to the main component at the chosen detection wavelength. The second of these choices is more typical, since determination of response factors may be done carefully on a one-time basis, whereas an assay requires a continuous supply of reference standard for independent

measurements accompanying each testing situation. Nonetheless, response factor determinations can be burdensome even if carried out in a traditional manner. A relatively pure reference standard of each substance of interest must be prepared, and each standard must be qualified with respect to its purity. Most commonly, this involves confirmation of (and correction for) HPLC homogeneity, and correction for moisture and/or residual solvents (both of which require separate measurement). Moreover, the amount of each reference standard must be sufficient such that an accurate weight may be measured as part of the overall RRF determination. Potential sources for error may be introduced if the isolated reference substance proves to be hygroscopic or unstable between the time it is qualified and the time its response factor is measured.

Use of qNMR as an orthogonal means to determine RRFs has significant advantages to address these shortcomings. While NMR has historically been used as a qualitative tool for structure determination, it is also inherently a quantitative technique since the integrated intensity of a proton resonance signal is directly proportional to the number of protons represented by that signal and the sample concentration. Moreover, all protons in a given spectrum are equally sensitive given the appropriate experimental setup. Hence, NMR provides an ideal mechanism by which to quantitate a given substance, assuming the association of a unique reporter proton resonance with the substance and the number of protons the resonance represents is known. Despite these clear advantages, qNMR remains a surprisingly unexplored approach in support of HPLC quantitation to many researchers.

QNMR DETERMINATION OF ANALYTE RRFS

The use of an internal reference standard that displays at least one proton resonance, which is distinct from all other proton resonances present in the spectrum, is critical to NMR quantitation. The internal reference standard should possess low volatility and be stable, of established purity, non-hygroscopic, and otherwise capable of being weighed with high accuracy. Examples include maleic acid or aspirin, both of which are available as USP reference standards.

An example test protocol for assaying the content of specific known substances in a sample could be as follows:

1. Accurately weigh an appropriate amount of the impurity calibration standard (10 to 100 mg) which contains both the main component and the known impurities of interest.
2. Accurately weigh between 10 - 15 mg of maleic acid (as the internal reference standard) into the same vial.
3. Add 500 mL d6-DMSO to the vial to dissolve both standards.
4. Acquire data under parameters that ensure complete relaxation of all protons.
5. Integrate the appropriate

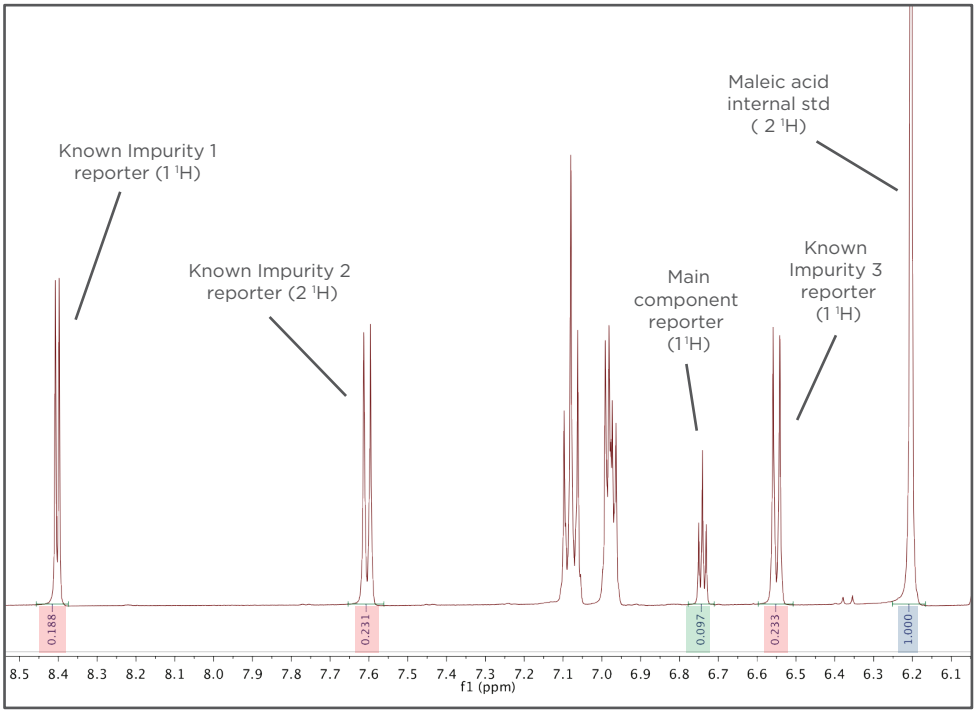


FIGURE 2: qNMR spectrum of an example impurity calibration standard containing unique reporter peaks of three impurities, a main component of interest, and maleic acid as the internal reference standard

reporter resonance peaks for both the maleic acid internal reference standard and for each of the various known substances of interest [Figure 2]. After correcting for the difference in molecular weights and the number of protons represented by each signal, ratio the corrected integrals and report the assay result for each substance [Table 1].

It is not necessary for the impurity calibration standard to be highly pure. It is only necessary that the chosen reporter peaks for each known substance, including the internal reference standard, are present in areas of the spectrum that are unique and unobstructed by resonances from other substances. The impurity calibration standard can be a solid, oil, or a concentrated solution possessing reasonably low volatility, so long as other resonance signals (including the NMR signal of the solvent) do not obscure the reporter resonances for the substances of interest.

Once the titer of each substance in the impurity calibration standard is determined, the RRFs of those substances in an HPLC method may be readily and precisely determined as follows [Figure 3]:

1. Accurately weigh an appropriate amount of the assayed impurity calibration standard into a suitably sized volumetric flask, and add the desired diluent to the mark, thereby making a solution with known concentrations of both the main component and its known impurities.

2. Analyze the impurity calibration standard with the chosen HPLC method that separates each impurity and the main component. For greater accuracy, repeat such that an average of data can be obtained.

3. Record the chromatographic area counts represented by the main component and for each of the respective known impurities of interest for each HPLC analysis.

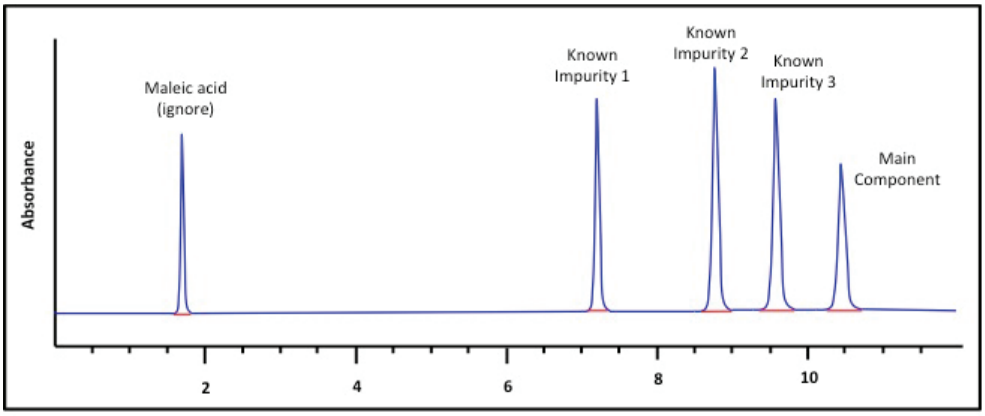
4. Calculate the number of counts/(mg/mL) for each substance using the titer values obtained previously [Table 1] for each substance using qNMR.

5. Calculate the ratio of counts/(mg/mL) for each known impurity to the counts/(mg/mL) value obtained for the main component, and report those respective numbers as the RRF for each known impurity [Figure 3].

Once an RRF for an impurity is determined, it can be routinely applied to the HPLC data, which allows the area percent values of

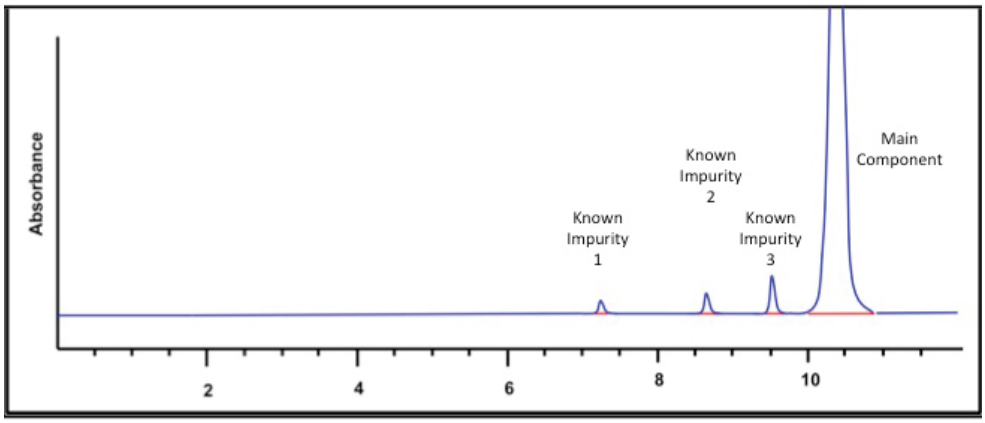
Substance	Reporter Peak Integral	Number of Protons Represented	Molecular Weight	Measured Weight (mg)	Total Volume of Dissolved Sample (mL)	Maleic Acid concentration (Internal Std, mg/mL)	Calculated Concentrations (corrected for proton number and MW, in mg/mL)
Maleic acid (Internal Std)	1.000	2	116.07	10.2315	0.500	20.463	--
Known Impurity 1	0.188	1	265.30	--	--	--	17.59
Known Impurity 2	0.231	2	297.30	--	--	--	12.11
Known Impurity 3	0.233	1	307.29	--	--	--	25.25
Main Component	0.097	1	325.31	--	--	--	11.13

TABLE 1: Example qNMR integral values and sample details yielding calculated titer values (in red)



Substance	Retention time (min)	Counts	mg/mL	Counts/mg/mL	Relative Response Factor
Known Impurity 1	7.3	1.11E+08	17.59	6.30E+06	0.82
KnownImpurity 2	8.8	1.33E+08	12.11	1.10E+07	1.43
Known Impurity 3	9.6	1.14E+08	25.25	4.53E+06	0.59
Main Component	10.5	8.54E+07	11.13	7.68E+06	1.00

FIGURE 3: LC/UV chromatogram of impurity calibration standard (top) and example calculation of Relative Response Factors based on titer values from Table 1 (bottom)



Substance	Retention time (min)	Counts	Area %	Relative Response Factor	Weight %
Known Impurity 1	7.3	1.47E+06	0.17	0.82	0.14
KnownImpurity 2	8.8	2.50E+06	0.29	1.43	0.41
Known Impurity 3	9.6	3.97E+06	0.46	0.59	0.27
Main Component	10.5	8.54E+08	99.08	1.00	99.17

FIGURE 4: LC/UV chromatogram of sample containing unknown amounts of known impurities (top) and example conversion of area percent into Weight % using Relative Response Factors (bottom)

each substance to be converted to a true assay value in weight percent [Figure 4]. As with traditional methods, it is important to recognize that response factor determination must be repeated any time a change is made to the HPLC method.

SUMMARY OF ADVANTAGES AND LIMITATIONS

Potential limitations of qNMR to determine RRFs include solubility of the internal reference standard, and the need to identify reporter resonances for each substance of interest that do not overlap with other resonances. These limitations can generally be overcome by changing the solvent and/or selection of an alternate internal reference standard.
The advantages of the approach outlined above for accurate impurity quantitation are:

1 RRF determination eliminates the costs associated with maintaining qualified impurity standards. Instead, an impurity calibration standard is used which contains the main component and each known impurity.

2 The impurity calibration standard can exist in a range of forms including solid, oil, or solution, so long as at least one recognizable reporter peak for each substance to be quantitated by qNMR remains unobscured by other resonances.

3 Once characterized, the impurity calibration standard may be stored in a freezer and reused if changes are made to the HPLC method. Note that this will require verification of impurity calibration standard titers prior to using the changed HPLC method.

REFERENCE

(1) Pauli GF, Chen S-N, Simmler C, Lankin DC, Gödecke T, Jaki BU, Friesen JB, McAlpine JB and Napolitano JG, Importance of Purity Evaluation and the Potential of Quantitative 1H NMR as a Purity Assay, J. Med. Chem., 57:pp 9220–9231, 2014