PHARMA

Compendial Identification Using Benchtop NMR: Example of Tobramycin According to Ph. Eur. 0645

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Innovation with Integrity

Nuclear Magnetic Resonance (NMR) spectroscopy is required for the first identification test of the aminoglycoside antibiotic tobramycin by the European Pharmacopoeia. This study demonstrates the straightforward implementation of this procedure using the benchtop Fourier 80 NMR spectrometer. The shortcomings of the existing procedure are discussed, along with possible strategies for enhancing robustness and reliability using the Fourier 80, without necessarily requiring additional validation efforts. The compact, cryogen-free design of the Fourier 80 facilitates the integration of NMR into standard Quality Control (QC) laboratory settings. It represents a modern analytical tool capable of addressing compendial procedures, such as the identification of tobramycin, while providing the flexibility to align with contemporary regulatory frameworks for improved qualitative and quantitative analytical procedures.

Introduction

Tobramycin is an aminoglycoside antibiotic derived from nebramycin and is produced by the fermentation of the actinomycete Streptomyces tenebrarius. First patented in 1965 and subsequently approved for medical use in the mid-1970's, tobramycin is effective against a broad spectrum of gram-negative bacteria. It is commonly prescribed to treat respiratory tract infections, particularly in cystic fibrosis patients because its efficacy against Pseudomonas aeruginosa. The drug is available in various formulations, including ophthalmic solutions, inhalation powders, and intravenous administrations.[1] The structure of tobramycin consists of a 2-deoxystreptamine ring linked to two amino sugars via glycosidic bonds (Figure 1). Similar to other aminoglycosides, tobramycin is highly hydrophilic. The basic nature of tobramycin results from the five hydroxyl groups and five amino groups, which have pKa values ranging from 5.67 to 9.29.[2]

These physicochemical properties, combined with the lack of UV-absorbing chromophores, complicate the analytical detection of tobramycin by traditional optical spectroscopies. For quantitative purposes, sophisticated and somewhat

[1] See for example R.N. Brogden et *al.* Drugs, **1976**, 12, 166–200, J.S. Elborn *et al.* Respiratory Medicine, **2022**, *192*, 106728 and associated references.

[2] T. da Silva Medeiros *et al.* Microchemical Journal, **2021**, *160*, 105658.

[3] See for example V. Manyangaetal *et al.* J. Pharma. Ana. **2013**, *3*, 161-167; V.P. Hanko et al., J. Pharma. Biomed. Ana., **2008**, *47*, 828-833.

tedious methods must be employed, typically involving liquid chromatography after derivatization and/or using less conventional detectors such as Evaporative Light Scattering Detector (ELSD) or Pulsed Amperometric Detection (PAD).[2,3] These challenges regarding chemical derivatization of the analyte prior to analysis and the dependence on reference material also extend to the qualitative identification tests found in pharmacopeias. For example, the United States Pharmacopeia-National Formulary (USP-NF) monograph for tobramycin necessitates identification by thin-layer chromatography (TLC) and a confirmation that the retention time of the test sample matches that of the reference in the assay test. Performing a standalone identification test is thus tedious because this assay involves reverse-phase liquid chromatography after a full derivatization of the analyte.

In contrast, the European Pharmacopoeia (Ph. Eur.) monograph 0645 of tobramycin provides a more straightforward approach as the first identification test of tobramycin requires only a single technique, Nuclear Magnetic Resonance (NMR) spectroscopy, while the second identification method [4] combines TLC and colorimetric testing. Indeed, by providing very detailed and specific information about an analyte, NMR spectroscopy does require additional confirmation technique while providing short turnaround times and minimal sample preparation. It is thus a method of choice for identification testing. When compared to infrared (IR) spectroscopy, a more traditional compendial identity testing technique that only informs on functional groups, the information from NMR is more detailed, unambiguous and ultimately specific since it is intrinsically linked to the exact chemical structure of the substance. Importantly, NMR is a universal method and does not require the systematic recording of reference data. Instead, experimental data can be directly compared to reference spectra or spectroscopic feature descriptions, which eliminates the need for reference material availability and management. This allows digital references, as introduced in the current draft of USP <11>, to be leveraged and drastically simplify the management of certified reference standard.[5] The unique ability of NMR to probe molecular structure has been exploited for decades in academic research and pharmaceutical discovery and development. It has been quickly established as the golden standard for structural analysis and applications for quantitative purposes are constantly growing.[6] The presence of NMR technology in Quality Control (QC) laboratories is however lagging, likely due in part to the required infrastructure, maintenance, and associated cost. Despite the early inclusion of NMR techniques in the general chapter of pharmacopeia, the number of NMR-based compendial tests is thus still limited. This paradigm is anticipated to change given the shift in focus to more state-of-the-art technology applied to compendial procedure observed in regulatory documents. Regulatory bodies indeed promote for better understanding of the analytical procedures through the modern concept of Analytical Quality by Design (AQbD), where the use of NMR is beneficial because of its data-rich, multi-attribute nature. In this instance, the recent revision of ICH[7] Q2(R2) significantly expanded the scope of analytical technologies for QC, which explicitly includes and exemplifies the use of NMR. The most recent drafts of the revised general NMR chapters of the USP-NF (<761> and <1761>) provide extended and highly detailed guidelines for the compendial use of NMR.

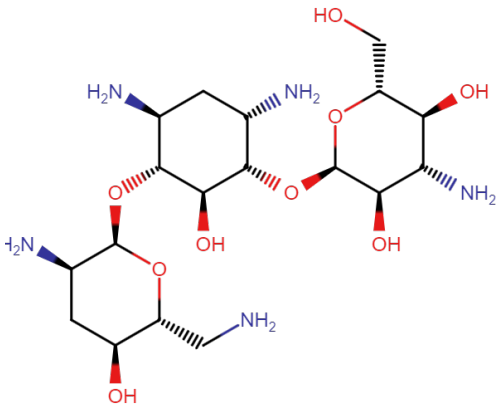

Figure 1: Chemical structure of tobramycin.

These recent publications from regulators and pharmacopeia organizations coincide with the growing popularity of benchtop NMR systems, such as the Bruker Fourier 80. These modern systems are well-suited for traditional QC laboratories or manufacturing environments because of their limited footprint and cryogen-free, permanent magnet configuration, which eliminates maintenance and significantly reduces cost. In a recent whitepaper,[8] we demonstrated the benefits of using the Fourier 80 to design modern, robust, yet flexible identification procedures as a way of streamlining identification testing by QC laboratories while leveraging AQbD concepts. In the present document, we will supplement these general considerations with the example of the Ph. Eur. tobramycin identification testing, exhibit the advantages of the Fourier 80 in implementing and performing this test, and describe further considerations to improve the robustness and confidence in the procedure

Implementation of the Compendial Procedure Using the Fourier 80

The NMR testing described in the Ph. Eur. 0645 for identification of tobramycin lacks detailed instruction because it only prescribes the analyte concentration (100 g/L), the solvent (deuterium oxide R) to be used, and "comparison" as the criteria. One could consider that details of the implementation of the procedure is the responsibility of the laboratory, based on the corresponding general chapter, but this may lead to different interpretations. An expert user will deduce that a comparison of the ^{1}H NMR spectrum of the test sample with that of the certified reference substance (CRS) is

[4] Second identification may be used in pharmacies, provided suitable traceability.

[5] As of August 2024.

[6] See for example G. F. Pauli *et al.* Anal. Chem., **2021**, *93*, 12162−12169.

[7] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use.

[8] V. Poirier et al. Streamlining Identification Testing for Quality Control Using Benchtop NMR, July 2024.

implied. Yet, other operators and nonexperts may be misled or confused because the nucleus to be recorded is not even specified. Furthermore, the Ph. Eur. general chapter about NMR (2.2.33) only gives few additional instructions for identification testing by merely stating 'the peaks in the two spectra or characteristic regions of the spectra should correspond in position, intensity and multiplicity". In our previous whitepaper, we discussed why such oversimplified approached are detrimental as they leave many variables to be selected by the laboratory and thus increase the risk of inadequate procedure and/or operator induced variability. In the modern paradigm of AQbD, this is even contradictory as risk-management and control of all possible variables is paramount. This is especially highlighted in the current draft of USP-NF <1761>: "critical quality attributes of the NMR spectrum should be identified, justified, and specified with numerical limits".

Figure 2 shows the overlay of representative ^{1}H NMR spectra of the tobramycin CRS and a commercial-grade source of tobramycin (Sigma T4014) both recorded on the Fourier 80 according to Ph. Eur. 0645 with identical acquisition and processing parameters. While the spectra are comparable to each other, small differences are noticeable:

- A slight change in the chemical shift of the glycoside proton at 5.2 ppm is observed.
- There are some changes in the shape of the resonances in the cluttered region at 4.2-2.5 ppm
- The CRS spectrum exhibits additional resonances at 0.9 and 0.1 ppm that are not observed in the commercial material.

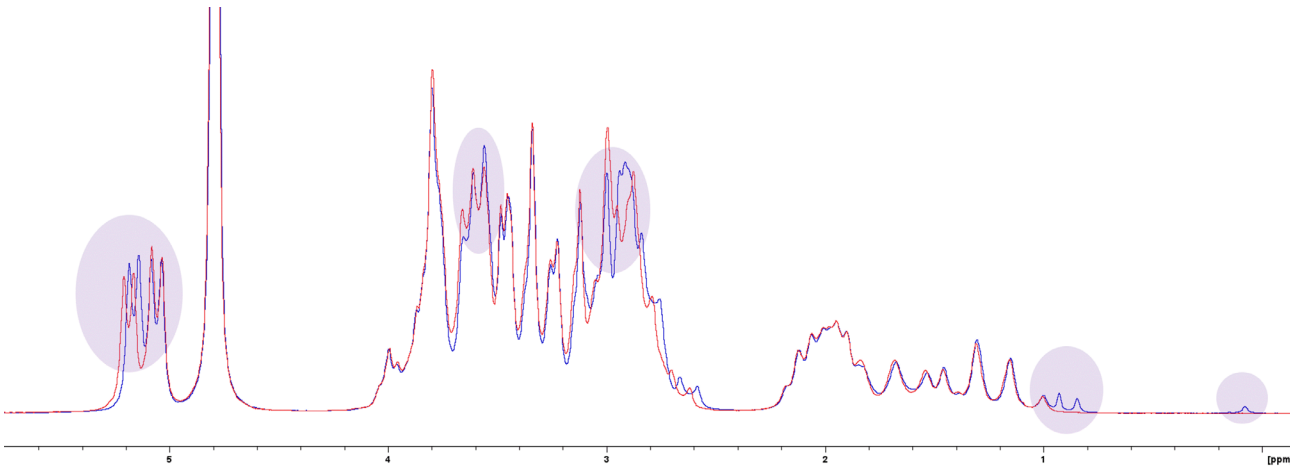

Figure 2: Overlay of the ^{1}H NMR spectra of tobramycin as recorded on the Fourier 80 according to Ph. Eur. 0645. The blue spectrum is that of the CRS while the red spectrum is a commercial-grade reference. The purple circles indicate areas where small differences are observed.

This leaves the conclusion of the test to the laboratory's discretion, based on its interpretation of these minor, yet detectable, discrepancies. Moreover, using a high-field NMR spectrometer does not resolve this uncertainty because even with the improved resolution on a 400 MHz spectrometer compared to the Fourier 80, the same differences are observable (Figure 3). This underscores that the Fourier 80's ability to detect subtle changes is not compromised by its lower resolution.

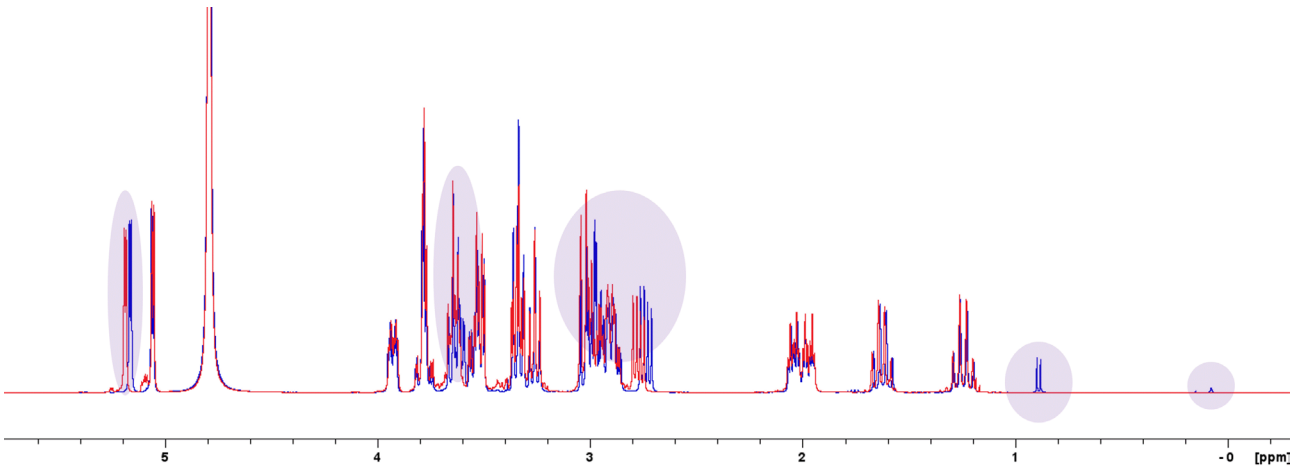

Figure 3: Overlay of the ^{1}H NMR spectra of tobramycin as recorded on a Bruker AVIIIHD 400 MHz NMR spectrometer according to Ph. Eur. 0645. The blue spectrum is that of the CRS while the red spectrum is a commercial-grade reference. The purple circle indicated areas where small differences are observable.

For an expert user, these differences are in fact not significant and can be easily explained by:

1) A general pH-dependency of the NMR resonances in water. Differences in pH induce structural changes in acido-basic sensitive species which noticeably affect their NMR spectra. This is particularly significant in the case of tobramycin, which contains five amino groups and therefore five different pKa. The small changes in chemical shifts and resonance shapes detected here are thus likely due to a slightly different pH in the unbuffered test solutions.

2) Contamination of the CRS by an aliphatic impurity and silicon grease resulting respectively in the 0.9 ppm and 0.1 ppm resonances. These are exogenous to tobramycin and should therefore not be considered when comparing the experimental data.

This scenario exemplifies why identification testing, although qualitative in nature, should not rely solely on qualitative and potentially subjective criteria but rather on clear and defined numerical acceptance criteria. It also underscores the importance of identifying all potential factors influencing the results during method design.[9] The contributions from pH differences, which can be critical, is probably not sufficiently controlled by the present procedure. While one might assume that a fixed-point concentration (100 g/L) could negate pH effects, NMR is highly sensitive to subtle changes in the chemical environment. Considering that the pH specification for Ph. Eur. grade tobramycin is 9.0 to 11.0 (as determined according to general chapter 2.2.3), this range is too broad to ensure perfectly reproducible chemical shifts and resonance shapes without additional buffering.

The risk analysis and method design for identification testing by NMR using the Fourier 80 are discussed in detail in our previous whitepaper.[8] In the present case, pH is an expected variable with a known effect that needs addressing during method design. Nonetheless, an identification procedure based on NMR should not aim at obtaining perfectly reproducible spectra. Instead, it should focus on identifying specific spectral features with defined numerical ranges to ensure a specific yet flexible test. This avoid tests result producing false negatives from imperfectly identical spectra. Based on these considerations, a set of criteria (Table 2) for the identification of tobramycin can be defined using the spectroscopic features of the CRS spectrum (Figure 4) reported in Table 1.

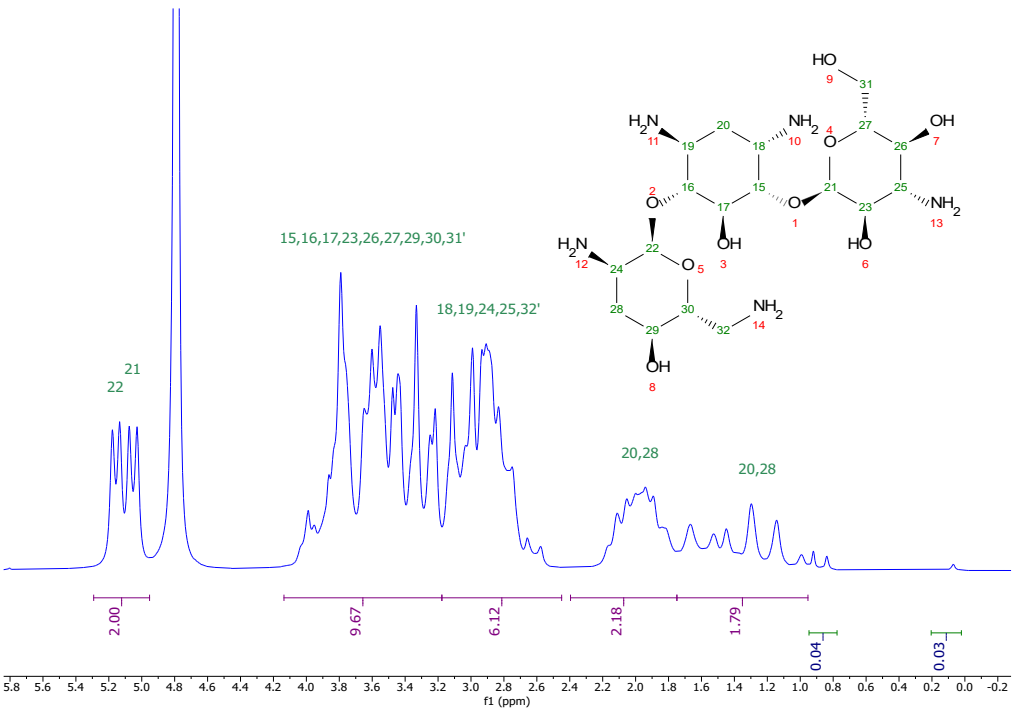

Figure 4: ^{1}H NMR spectrum of tobramycin CRS (100 g/L, D_2O, 25°C) at 80 MHz with the corresponding structural assignments.

[9] See reference 8 for a detailed discussion about risk-assessment for identification testing by NMR.

Signal	Attribution	Shift (ppm)	Integral	Multiplicity[a]	Coupling constant (Hz)
1	20,28	0.95-1.76[c]	2	m	/
2	20,28	1.76[c]-2.40	2	m	/
3	18,19,24,25,32	2.45-3.20[c]	6	m	/
4	15,16,17,23,26,27,29,30,31	3.20[c]-4.20	10	m	/
5	21	5.05	2[b]	d	3.7
6	22	5.15		d	3.9

a) m: multiplet or mix of resonances. b) relative integration reference set to 2. c) no return to the baseline

Table 1: ^{1}H NMR spectroscopic feature in tobramycin CRS (100 g/L, D_2O, 25°C) as recorded on a Fourier 80.

Signal	Attribution	Shift (ppm)	Integral	Multiplicity[a]	Additional criteria
1	20,28	0.95-1.76[c]	2	m	Pseudo quadruplet shape on the right side of the area which two main resonances are 12.3 ± 0.5 Hz apart.
2	20,28	1.76[c]-2.40	2	m	/
3	18,19,24,25,32	2.45-3.20[c]	6	m	/
4	15,16,17,23,26,27, 29,30,31	3.20[c]-4.20	10	m	/
5	21	5.05	2[b]	d	Coupling constant of 3.7 ± 0.5 Hz
6	22	5.15		d	Coupling constant of 3.9 ± 0.5 Hz

a) m: multiplet or mix of resonances. b) relative integration reference set to 2. c) no return to the baseline

Acceptance criteria: Resonances in this table must be detected at the reported chemical shift or within the chemical shift range for non-resolved multiplet or mix of resonances with a 0.05 ppm tolerance. Relative integration values and multiplicity must match the reported values, using global integration of resonances 5 and 6 as relative integration reference set to 2. Chemical shift referencing must be done using the HDO resonance set at 4.79 ppm. Any other resonance detected with a relative integration above 0.5 should be reported as a potential contaminant and investigated.

Table 2: Example of acceptance criteria for the identification of tobramycin by ^{1}H NMR using the Fourier 80.

Due to the complex spectrum of tobramycin, defining and detailing discrete multiplet features is not feasible nor practical. Nevertheless, the set of criteria outlined in Table 2 provides 16 unique spectroscopic features to be matched with defined parameters, ensuring a highly specific procedure while allowing the necessary flexibility. The tolerances are based on general considerations regarding NMR identification procedures[8] and take into account potential pH effects. The robustness of the test could be further improved by testing the two extreme pH values allowed for tobramycin in solution (9.0 to 11.0) to fully assess the impact on these spectroscopic features and ensure that the criteria in Table 2 does not lead to false negative results. Nonetheless, the commercial grade of tobramycin tested here (represented by the red spectrum in Figure 2) would successfully pass the identification test based on these example criteria and avoids leaving the interpretation of subtle spectral differences to the operator.

For an identification test, it is also crucial to consider a specific procedure that distinguishes the target compound from closely related structures that may be present during manufacturing or in the supply chain. As an example, the set of criteria defined in Figure 2 was challenged against a known impurity of tobramycin with a very similar structure: kanamycin B. This related substance differs from tobramycin by only a single oxygen atom (Figure 5), which minimally affects the physicochemical properties and can be challenging to detect using traditional compendial identification techniques such as IR and TLC.

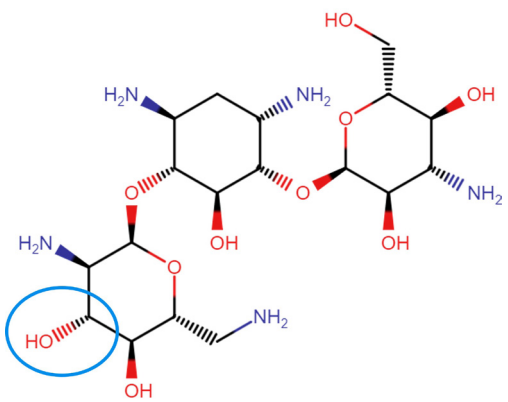

Figure 5: Chemical structure of kanamycin B. The blue circle highlights the additional hydroxyl group compared to tobramycin.

By contrast, NMR is the ideal analytical tool to selectively detect such structural changes. Altering a glycoside CH_2 group to CH(OH) group has profound consequences on the NMR spectrum because of its sensitivity to chemical environments. This is illustrated in Figure 6 which shows a representative ^1H NMR spectrum of the sulfate salt of kanamycin B recorded in the same condition as in Ph. Eur. 0645 using the Fourier 80 and compared to that of tobramycin.

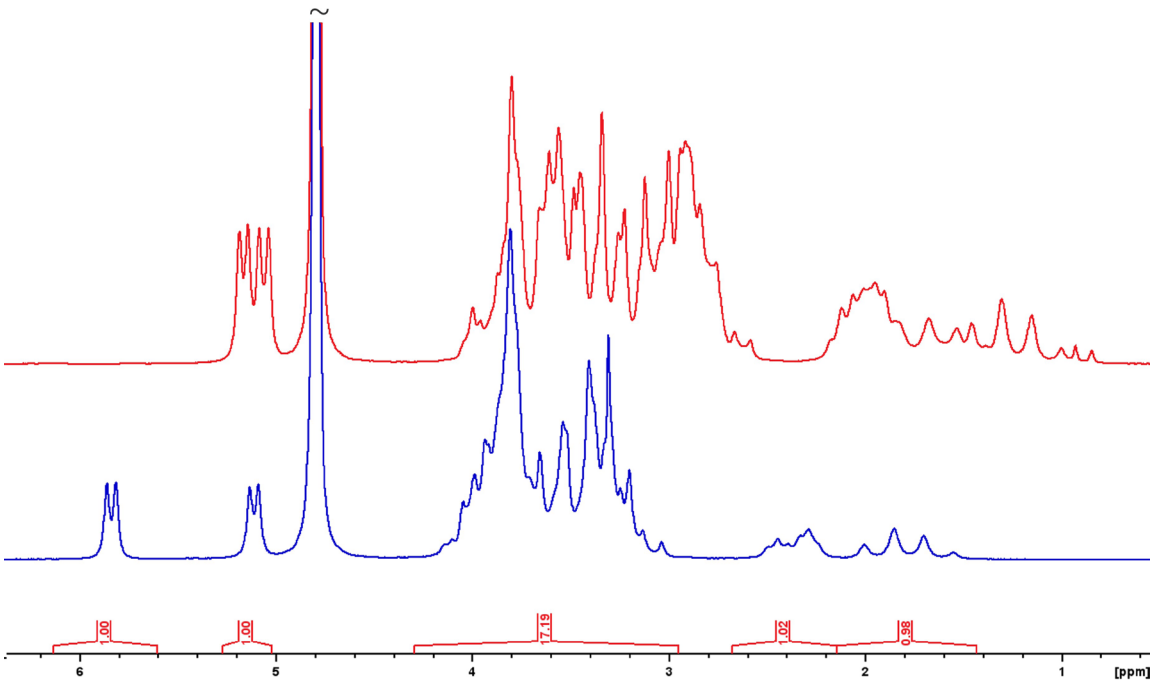

Figure 6: Overlay of the ^1H NMR spectra of the sulfate salt of kanamycin B (blue) and of obramycin (red) an as recorded on a Fourier 80 (100 g/L, D_2O, 25°C). Integration values in red are relative to the kanamycin B spectrum.

Large differences in the spectral features of kanamycin B are unambiguously identifiable when compared to tobramycin and the criteria defined in Table 2 would not match any of the signals observed. This is due to a combination of two effects:

- Kanamycin B was recorded in acidic conditions (sulfate salt), evidencing again the strong effect of pH on the NMR spectrum.
- Kanamycin B has one less non-labile proton[10] than tobramycin and only one CH_2 group. This has immediate consequences on the resonance chemical shifts and integrations. First, the relative integration of the entire spectra for Kanamycin B measures 21 protons whereas the integration of the tobramycin spectrum measures 22 protons. Second, in the high field area below 3 ppm, the integration measures only 2 protons in Kanamycin B from its single CH_2 group while 4 are observed in tobramycin.

Therefore, the criteria defined in Table 2 are detailed enough to ensure a very specific procedure and discriminate related substances with very similar structure. Because the instructions of Ph. Eur. 0645 for the NMR identification testing are very limited, such supplemental strategy would not constitute a deviation from the compendium. It could be implemented without being considered as an alternative procedure while providing more formal criteria for the identification test. However, the systematic recording of the reference data on the CRS would still be required as this is explicitly mandated in the monograph. Additionally, better control of the variable pH using for example a deuterated buffer would be beneficial but considered a deviation as Ph. Eur. 0645 stipulates D_2O. For QC laboratories seeking to

[10] Labile protons are not detectable by ^1H NMR when using D_2O as the solvent.

routinely perform this test, it might thus be advantageous to develop and validate an alternative procedure using the Fourier 80 based on the considerations above for a more streamlined and robust analytical workflow compared to the original monograph test.

Finally, the versatility of NMR, both for high-field instruments and benchtop systems like the Fourier 80, provides an analytical toolbox to many applications. It can be easily leveraged in case of Out Of Specification (OOS) results and/or for regulatory investigation purposes. For instance, structural investigations using a combination of one-dimensional and multi-dimensional NMR experiments are straightforward to perform. A detailed discussion of this level of characterization is beyond the scope of the present manuscript but is illustrated in Figure 7. In this example and using the same sample preparation as for the identification test, the detection of all non-quaternary carbons of tobramycin is achievable through analysis of a ^{1}H-^{13}C correlation map (so called ^{1}H-^{13}C edited-HSQC) collected in less than 30 minutes using the standard parameters defined in the software (Topspin). This demonstrates the versatility of the Fourier 80 in providing an invaluable qualitative investigational tool to QC laboratories that uses the same piece of equipment as routine tests and without specific conditioning.

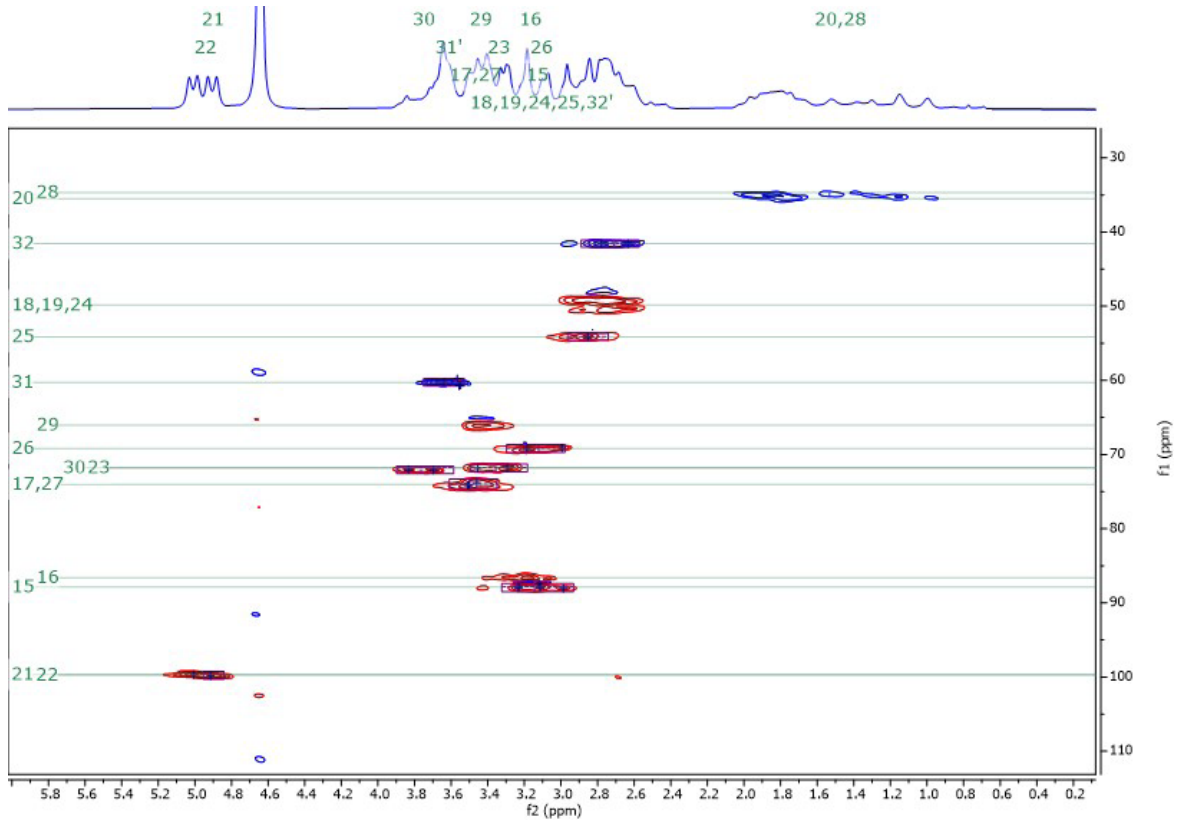

Figure 7: ^{1}H-^{13}C Edited HSQC (hsqcedetgp, NS 16, 256 t1 increments with NUS set to 30%) of tobramycin recorded on a Fourier 80 (100 g/L, D_2O, 25°C) allowing the indirect detection of all non-quaternary carbons. Structural attribution refers to Figure 4 and was performed combining HSQC/HMBC/COSY experiments recorded on a Fourier 80 and chemical shift prediction tools.

Conclusion

In this whitepaper we have demonstrated the successful application of the benchtop Fourier 80 NMR spectrometer for the identification test of tobramycin according to Ph. Eur. 0645. By leveraging previous published knowledge of NMR identification testing, a strategy for improving the robustness of the procedure without compromising the compliance to the current monograph was possible using the Fourier 80. Identification testing such as this example serves a simple yet important purpose and so a descriptive, detailed procedure is critical to ensure robust, reliable procedure and avoid operator induced variability. These general considerations stand true regardless of the analytical technology, but are significantly easier to execute using NMR. The above example demonstrates that NMR offers a very specific, yet universal identification tool, even in the case of the analytically challenging substance like tobramycin. The Fourier 80 is a perfect tool for QC laboratories to adopt NMR, providing the benefits of this technology in a compact, cryogen-free system with full automation capabilities and build-in support for GMP compliance.

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