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^{1}H and ^{19}F NMR in drug stress testing: the case of voriconazole†

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Stress tests form an important part of drug development, and of subsequent accreditation. Commonly the change in chemical composition over time is determined using chromatographic methods that separate the mixture components, but when structural characterization of the degradation products is required, other methods are needed. Arguably the most powerful method for determining chemical structure information is NMR spectroscopy. Here we show that NMR alone, using a combination of simple ^{19}F, ^{1}H, and DOSY experiments, can characterise both structure and kinetics of an intact reaction mixture, without the need for any physical separation.

Introduction

During the development of a drug, and certainly before its release to the market, it is important to have information about its stability under various conditions, since this affects both its safety and its efficacy.[1] This information is commonly found by stress testing (forced degradation), *i.e.* subjecting the drug to harsh conditions to infer information about its long-term stability. Typical examples in solution include acid, alkaline and oxidative conditions, as well as high temperature.[1] Monitoring the reaction time course under such conditions provides kinetic and structural information on the reagents, intermediates, and final products, improving the understanding of reaction mechanisms and kinetic properties.[2] Common spectroscopic methods used for this purpose include mid-infrared (MIR),[3] Raman,[4] near-infrared (NIR),[5] mass (MS)[2,6] and nuclear magnetic resonance (NMR)[7–12] spectroscopies.

The choice of technique depends on the target reaction and on what information it is required. NMR has some distinct advantages over other techniques, since it provides very detailed information about chemical structure, and it is non-destructive. However, it also has the disadvantage of low sensitivity, and it is far from ideal for mixtures, because of the difficulty, common to many spectroscopic methods, of assigning signals to individual species. Diffusion-ordered spectroscopy (DOSY),[13,14] in which the signals from mixture components are differentiated according to the diffusion behaviour of the species involved, has been shown to be effective in the analysis of drug mixtures.[15–17] An alternative approach is to use HPLC-NMR, but this involves physical separation and often requires lengthy chromatographic optimization. DOSY, in contrast, keeps the sample intact, so that interactions can be studied, and sample recovery is trivial; it can be performed on standard NMR equipment, present in most chemistry departments and pharmaceutical industry laboratories worldwide. NMR can also provide specific information about different nuclei and isotopes, which is a great advantage for fluorinated drugs. A large proportion of modern drugs, that has steadily increased over the last decade,[18,19] contain at least one fluorine atom. ^{19}F is an excellent probe in NMR, because of its strong signal, high sensitivity to changes in chemical environment, and excellent spectral resolution due to its wide chemical shift range.

In a previous study using HPLC, only one of the voriconazole degradation products could be characterized, while the second degradation product was reported to be an unstable component that degrades into multiple species, including the degradation product already identified, during the solvent evaporation step.[20] In another study Ahmed and Abdalla described the development of an HPLC methodology to isolate voriconazole and its degradation products; apart from being time-consuming to develop and validate, at the end of the process none of the products identified by the method were structurally characterized.[21] Such characterization is today a regulatory requirement, since more importance is attached to the structures and to the potential toxicity of degradation products.

NMR is extensively used in pharmaceutical analysis, in all stages of the development and production of active pharmaceutical ingredients. This includes the identification, characterization, and quantification of species that arise during the manufacturing process or are generated during storage, and quality assessment of drug formulations.[12,15–17,22] This article illustrates a less common application, the use of ^{1}H and ^{19}F NMR for stress testing. An alkaline stress test of the fluorinated drug voriconazole is performed entirely inside a standard NMR

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tube, without the need for separation. The two main degradation products are characterized, one of them for the first time, and a complete kinetic profile is produced.

Experimental section

Full ^{1}H, ^{13}C and ^{19}F spectral assignments of voriconazole (Fig. 1) were performed, using 1D and 2D NMR experiments, prior to degradation tests (see Fig. S2–S12 in ESI†). ^{1}H and ^{13}C assignments were in agreement with those previously reported;[19,23,24] unequivocal coupling constant assignments and ^{19}F NMR assignments are given here for the first time.

Voriconazole assignments

^{1}H NMR (600.17 MHz, CD_3OH): δ (ppm) 9.00 (1H, d, $^{5}J_{H2''F5''}$ = 2.40 Hz, H2″); 8.75 (1H, d, $^{3}J_{H6''F5''}$ = 1.86 Hz, H6″); 8.27 (1H, s, H5‴); 7.59 (1H, s, H3‴); 7.49 (1H, td, $^{3}J_{H6'H5'}$ = 9.01, $^{4}J_{H6'F2'}$ = 9.01 and $^{4}J_{H6'F4'}$ = 6.67 Hz, H6′); 6.97 (1H, ddd, $^{3}J_{H3'F2'}$ = 12.12, $^{3}J_{H3'F4'}$ = 8.82 and $^{4}J_{H3'H5'}$ = 2.52 Hz, H3′); 6.86 (1H, td, $^{3}J_{H5'F4'}$ = 8.04 Hz, H5′); 6.39 (1H, s, OH); ~4.85* (1H, d, H1); 4.36 (1H, d, $^{2}J_{H1H1}$ = 14.28 Hz, H1); 4.16 (1H, q, $^{3}J_{H3H4}$ = 6.84 Hz, H3) and 1.14 (3H, d, H4). ^{13}C NMR (125.69 MHz, CD_3OH): δ (ppm) 164.36 (1C, dd, $^{1}J_{C4'F4'}$ = 248.2 and $^{3}J_{C4'F2'}$ = 12.6 Hz, C4′); 160.44 (1C, dd, $^{1}J_{C2'F2'}$ = 246.5 and $^{3}J_{C2'F4'}$ = 11.9 Hz, C2′); 160.04 (1C, d, $^{2}J_{C4''F5''}$ = 12.6 Hz, C4″); 157.74 (1C, d, $^{1}J_{C5''F5''}$ = 264.1 Hz, C5″); 154.89 (1C, d, $^{4}J_{C2''F5''}$ = 7.7 Hz, C2″); 151.02 (1C, s, C3‴); 146.58 (1C, d, $^{2}J_{C6''F5''}$ = 22.7 Hz, C6″); 145.90 (1C, s, C5‴); 131.80 (1C, dd, $^{3}J_{C6'F2'/4'}$ = 9.23 and $^{3}J_{C6'F2'/4'}$ = 5.88 Hz, C6′); 125.36 (1C, dd, $^{2}J_{C1'F2'}$ = 12.29 and $^{4}J_{C1'F4'}$ = 3.75 Hz, C1′); 112.14 (1C, dd, $^{2}J_{C5'F4'}$ = 20.88 and $^{4}J_{C5'F2'}$ = 2.59 Hz, C5′); 105.02 (1C, t, $^{2}J_{C3'F2'/4'}$ = 27.26 Hz, C3′); 78.70 (1C, d, $^{3}J_{C2F2'}$ = 4.92 Hz, C2); 58.08 (1C, d, $^{4}J_{C1F2'}$ = 4.86 Hz, C1); 39.11 (1C, d, $^{3}J_{C3F5''}$ = 4.95 Hz, C3) and 15.51 (1C, s, C4). ^{19}F{^{1}H} NMR (470.35 MHz, CD_3OH): δ (ppm) −137.30 (1F, s, F5″); −112.92 (1F, d, $^{4}J_{F4'F2'}$ = 8.3 Hz, F4′) and −109.45 (1F, d, F2′). *Assigned from ^{1}H–^{13}C HSQC.

Voriconazole degradation

A European Pharmacopoeia Reference Standard of voriconazole was purchased from Sigma-Aldrich. The alkaline stress test was performed in methanol-d_3, because of the good voriconazole solubility in, and the solvent stability to, alkali. Methanol-d_3 was preferred to methanol-d_4 in order to avoid losing exchangeable hydrogens (OH and NH in this case). There is no consensus in the literature as to what concentration of alkali to use for such a stress test;[1,25,26] here voriconazole and NaOH were used in the proportion 1 : 2 (24 mM : 48 mM) in methanol-d_3 at 10 °C. The alkali concentration most commonly used in stress testing is 0.1 M,[1] under such conditions voriconazole degrades very rapidly, so a slightly lower concentration was used here in order to obtain a convenient degradation rate with sharp ^{19}F NMR signals. Acid and thermal stress tests were also performed, but no degradation was seen in the first, and for the thermal degradation the kinetics differed between different batches of DMSO-d_6 and voriconazole (see ESI†). All NMR experiments were performed using a Bruker AVANCE III spectrometer operating at a 500 MHz ^{1}H frequency, equipped with a 5 mm BBO probe; full experimental data and all experimental parameters can be downloaded from DOI: 10.17632/9pc5pyrp7c.1. In order to acquire quantitative NMR data, the recovery delay (d1) was set to 20 s, 5 times the largest T_1 measured for the compounds of interest.

^{1}H DOSY with solvent suppression

^{1}H DOSY data were acquired using the Oneshot45 experiment[27,28] (Bruker code is available *via* the following link: http://nmr.chemistry.manchester.ac.uk/?q=node/264) with presaturation of the hydroxyl residual proton signal of methanol-d_3. The diffusion experiment was performed using a sample in a 3 mm NMR tube,[29,30] to minimize convection, with a net diffusion-encoding gradient pulse width (δ) of 2.4 ms, a diffusion delay (Δ) of 60 ms, and sixteen nominal gradient amplitudes ranging from 4.8 to 38.5 G cm^{-1}. The delay for gradient recovery (d16) and duration of the gradient purge pulse (p19) were fixed at 0.2 and 0.6 ms, respectively. The experiments were carried out at a nominal probe temperature of 10 °C, and the nominal power level used for presaturation was 84 μW. ^{19}F DOSY data were acquired using the Oneshot pulse sequence,[13] with the same parameters as for the ^{1}H DOSY spectrum. The processing of DOSY experiments was carried out using the free DOSY Toolbox.[31] ^{1}H DOSY processing used a 32k data table, phase correction, baseline correction, and reference deconvolution with a 3 Hz wide Lorentzian target lineshape. ^{19}F DOSY processing was identical except for a 10 Hz Lorentzian target lineshape.

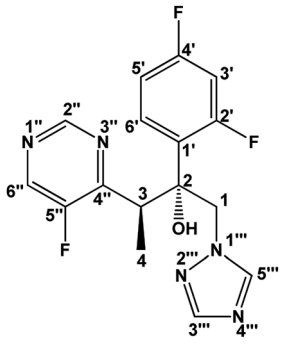

Fig. 1 The structure of voriconazole, $C_{16}H_{14}F_3N_5O$, molar mass of 349.12 g mol^{-1}. ^{1}H, ^{13}C, and ^{19}F assignments are given below.

Results and discussion: voriconazole alkaline degradation

Voriconazole is relatively sensitive to alkali, leading to rapid degradation at room temperature, so the study was performed at 10 °C to allow appropriate time resolution for the reaction kinetics. From the ^{1}H NMR spectra, it was clear that virtually all voriconazole had been degraded after 17 h as only signals from degradation products, one of which showed some rather broad signals (Fig. 2), were seen. The ^{19}F NMR spectra (Fig. 3) showed clearly that at least three new signals related to the major degradation product(s) appeared.

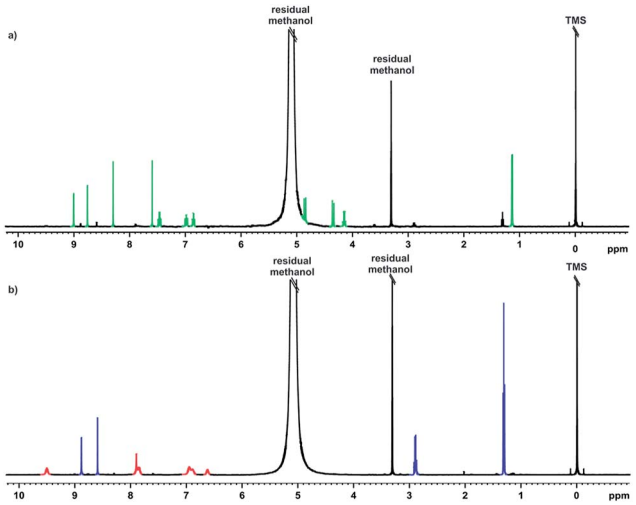

Fig. 2 ^{1}H NMR spectra of alkaline stress testing; (a) time 0, and (b) after 17 h. Signals shown in green are from voriconazole, in red from degradation product 1, and in blue from degradation product 2, both here and in subsequent figures.

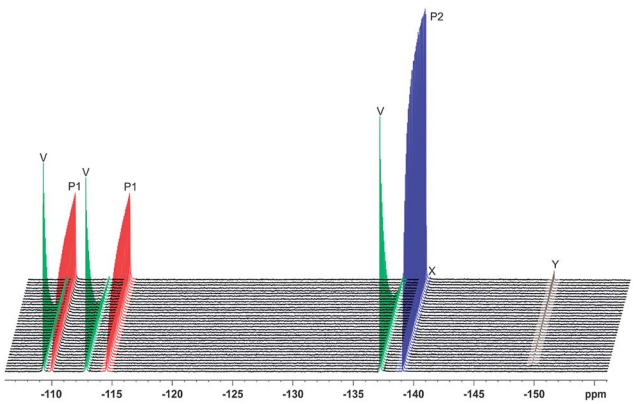

Fig. 3 ^{19}F NMR spectra during a 17 h alkaline stress test, showing the consumption of voriconazole (V), the formation of major degradation products 1 (P1) and 2 (P2), and minor degradation products X and Y. Spectra are shown at half hour intervals.

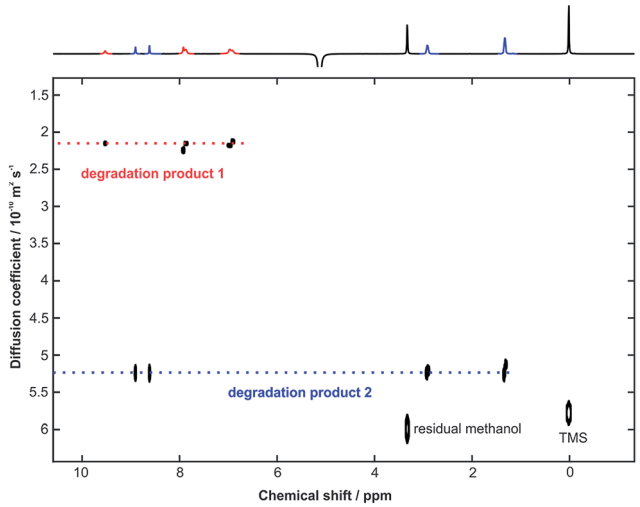

Fig. 4 500 MHz ^{1}H DOSY spectrum, with the least attenuated 1D spectrum shown at the top, acquired after 17 h of monitoring of a sample containing 24 mM voriconazole, and 48 mM NaOH in CD_3OH at 10 °C.

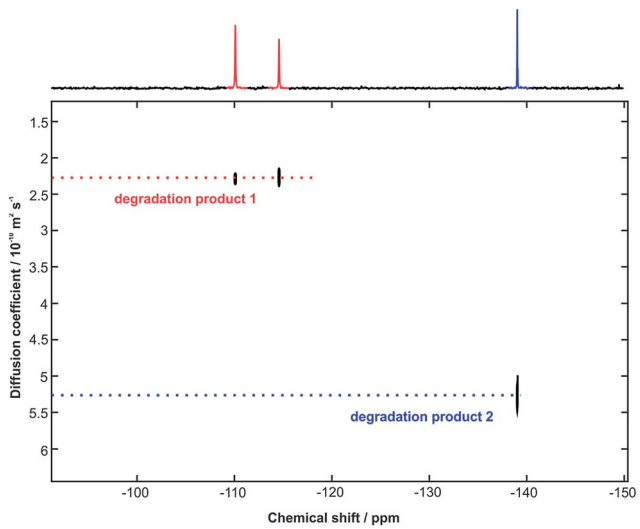

Fig. 5 470 MHz ^{19}F DOSY spectrum, with the least attenuated 1D spectrum shown at the top, acquired immediately following the ^{1}H DOSY experiment of Fig. 4.

The ^{1}H and ^{19}F DOSY experiments recorded at the end of the stress test (Fig. 4 and 5) showed that two main degradation products were generated, and that both are fluorinated. The agreement of diffusion coefficients between the experiments makes it easy to correlate the proton and fluorine spectra for each component. Degradation product 1 shows a lower diffusion coefficient (*i.e.* product 1 is a larger compound) and has two fluorine resonances, while degradation product 2 is smaller and has a single fluorine resonance.

The alkaline stress testing showed two main degradation products. However, expansion of the ^{19}F NMR spectrum (Fig. 6) revealed the presence of two minor products (X and Y). Degradation product 1 is in exchange between keto and enol tautomers, with the latter form favoured under alkaline conditions. This explains the broad extra signals observed in the ^{19}F spectrum, which arise from the disfavoured keto form.

^{19}F NMR spectra were acquired every 10 min during the reaction time course, and the signal integrals *versus* reaction time course were fitted (Fig. 7) to the first order kinetic scheme of Fig. 8. The voriconazole degrades into products 1 and 2 with a rate constant of 0.27 h^{-1}; product 1 is present in keto and enol forms that are in an equilibrium that is slow on the chemical shift timescale but fast on the timescale of reaction ($k_{\text{keto}\rightarrow\text{enol}} \sim$ 70 s^{-1}), while product 2 decays further to two minor unidentified degradation products, X and Y with first order rate constants of 0.0085 and 0.0028 h^{-1}, respectively (Fig. 8). Confirmation of the structures of products 1 and 2 is provided in the ESI.†

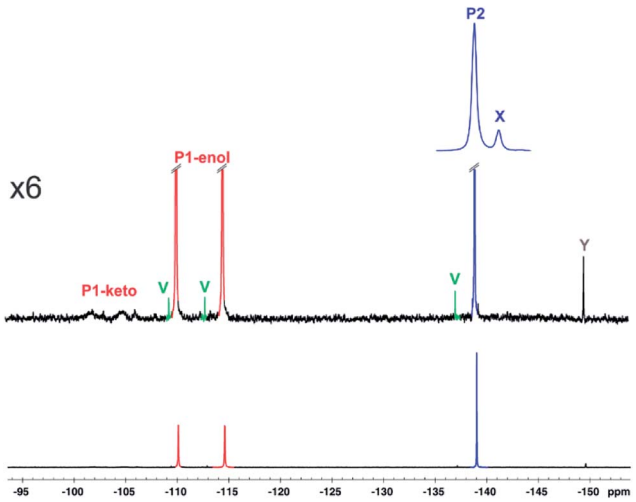

Fig. 6 Expansion of ^{19}F NMR spectrum acquired after 24 h with the same sample as Fig. 4.

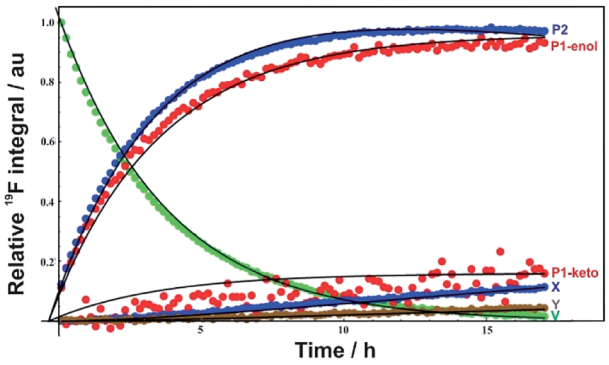

Fig. 7 Voriconazole degradation profile under alkaline conditions, showing the relative concentrations of voriconazole (V), major degradation products 1 (P1) and 2 (P2), and two minor degradation products (X), (Y). Points show experimental measurements, solid lines the result of fitting to the kinetic scheme shown in Fig. 8.

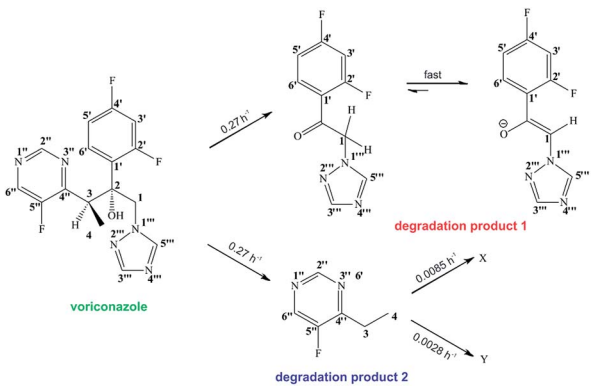

Fig. 8 Proposed pathway for alkaline degradation of voriconazole.

Conclusion

The main purpose of this article is to illustrate the utility of NMR as a tool in monitoring drug degradation without the need for physical separation. The advantages of NMR are particularly marked for fluorinated drugs, since ^{19}F shows excellent spectral resolution because of the sensitivity of its chemical shift to local environment. ^{1}H and ^{19}F DOSY spectra are helpful in providing information on the number and nature of compounds involved in the degradation process. Here a detailed analysis of the reaction time course as followed by ^{19}F NMR revealed the presence of minor degradation products, and allowed us to identify and quantitate a pathway for the alkaline degradation of voriconazole that offers very good agreement with experimental data. The identities and assignments of the major degradation products were determined as described in the ESI,† with the structure of degradation product 2 being reported for the first time.

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