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Impurity profiling in bulk pharmaceutical batches using ^{19}F NMR spectroscopy and distinction between monomeric and dimeric impurities by NMR-based diffusion measurements

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Abstract

The impurity profile of production batches of fluorine-containing drugs can be characterised efficiently using ^{19}F NMR spectroscopy. This yields the number and proportions of impurities in the bulk drug to a level of ≈ 0.1 mole% in a few minutes of NMR experiment time. The approach has been exemplified using a partially purified batch of the steroidal product fluticasone propionate, the impurities in which include a number of dimeric species. Further distinction between the monomer and dimer impurities has been achieved through high resolution chemical shift-resolved NMR measurement of molecular diffusion coefficients on the intact mixture using ^{19}F NMR spectroscopy. The ability of NMR-based diffusion coefficient determination to distinguish between monomeric and dimeric substances was validated using a standard mixture of authentic materials containing both monomers and dimers.

Keywords: NMR; ^{19}F; Diffusion; Impurity; Fluticasone; Production

1. Introduction

The manufacture and quality control of a drug product is controlled by a variety of national regulatory authorities. In addition to the need to prove drug efficacy, there is also a strong empha-

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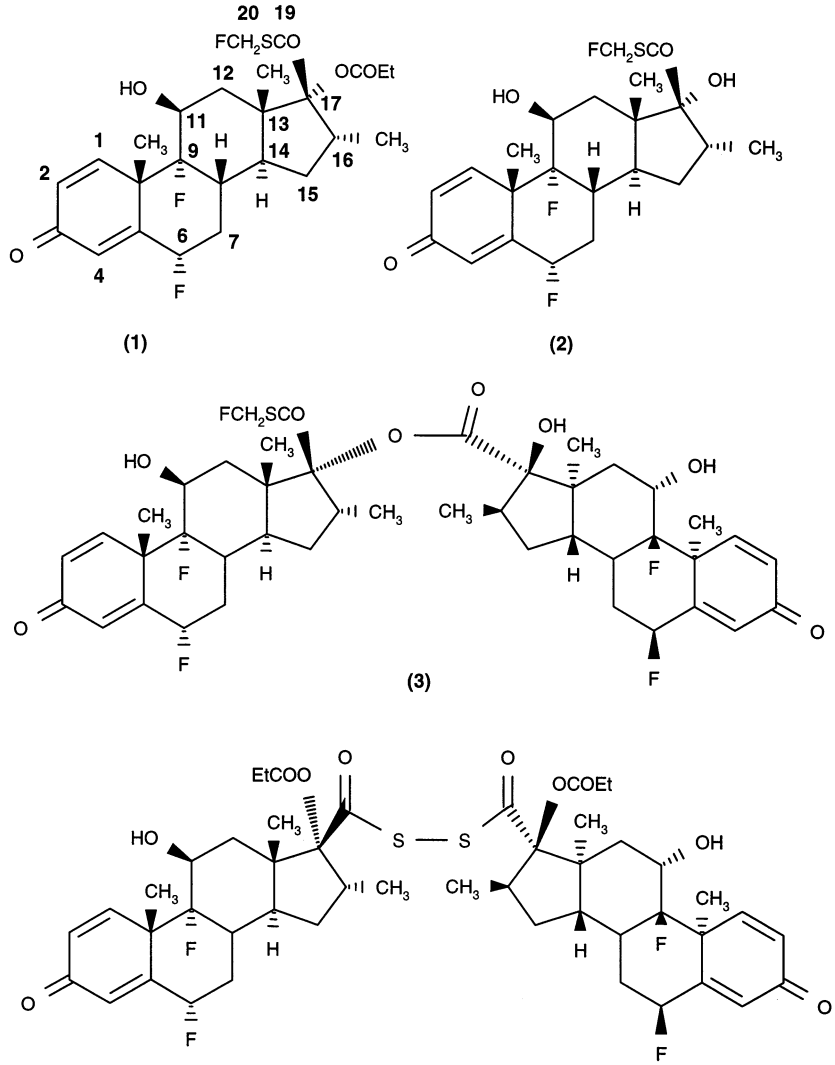

Scheme 1. Structures of fluticasone propionate **(1)** and related model monomer and dimer compounds. The atom numbering is as shown.

sis on the purity of final drug substances and registration authorities require full characterisation and identification of any impurities at the level of 0.1% of the UV peak area using HPLC [1]. Currently, in order to characterise such impurities, it has proved necessary to isolate individual components by preparative HPLC and use NMR spectroscopy and mass spectrometry for structural identification. This work is often time consuming and expensive but even so may not always be conclusive. We recently showed that directly-coupled HPLC–NMR spectroscopy can provide a more efficient method for this type of study and this has recently been applied to characterise a number of impurities in a partially purified batch of fluticasone propionate **(1)** which has the chemical structure shown in Scheme 1 [2]. There is, however, a considerable need to develop and validate new methods for determining product purity. With this aim, we have now applied NMR spectroscopy to the measurement of molecular diffusion coefficients of the mixture components in a

partially purified batch of **(1)** to provide a distinction between monomeric and dimeric substances without the need for HPLC separation.

High resolution ^{19}F NMR spectroscopy is potentially an excellent method for product profiling for substances containing fluorine since the C–F bond is strong and degradative defluorination is relatively rare. In addition, it is likely that any related impurities or degradation products of the drug will also contain fluorine. The ^{19}F nucleus is 100% abundant, with spin = 1/2, and a large magnetic moment [3] which results in ^{19}F NMR spectroscopy being a very sensitive method of detecting minor fluorine-containing compounds in a bulk production sample of a pharmaceutical material. It is possible to use ^{19}F NMR spectroscopy as a simple way of determining the number of different fluorine-containing components that are present in a mixture. This is achieved by counting the number of different fluorine peaks in a spectrum around a specific chemical shift region and this is easier for ^{19}F NMR than for ^{1}H NMR as usually there are fewer fluorine atom environments present, the ^{19}F chemical shift range is wider than that for ^{1}H NMR and each chemically distinct ^{19}F nucleus in a ^{1}H-decoupled spectrum usually gives rise to only a single resonance. Provided that the ^{19}F NMR spectrum is acquired under conditions of full T_1 relaxation, it is possible to quantify the relative amounts of the different components in the mixture by measuring integrals of the minor fluorine peaks in the spectrum.

The diffusion coefficient is a whole molecule property which reflects the molecular size and thus this may provide a new approach for initial characterisation of components in complex mixtures. The measurement of diffusion coefficients using NMR spectroscopy is based on a pulsed field gradient spin-echo experiment and problems associated with this method were addressed by Johnson et al. [4] who developed the longitudinal eddy current delay (LED) method. This allowed diffusion coefficients to be determined for each resonance in a high resolution NMR spectrum and the term diffusion-ordered spectroscopy (DOSY) [4] was introduced where the spectrum is plotted as a pseudo-two-dimensional contour representation with the chemical shifts on the horizontal axis and the derived diffusion coefficients on the vertical axis. The DOSY approach has been applied to a number of mixtures including cell extracts, [5] biofluids [6,7] and protein–drug binding [8]. In the present study, the measurement of molecular diffusion coefficients using ^{19}F NMR spectroscopy has been used to distinguish monomeric from dimeric species in a partially purified batch of bulk drug substance.

2. Experimental

The test sample of fluticasone propionate **(1)** was obtained from a partially purified batch of the drug substance, prior to formulation, from GlaxoWellcome, Montrose, UK. In addition, authentic samples of fluticasone propionate itself **(1)**, a related monomeric compound **(2)**, and two related dimeric compounds **(3)** and **(4)** were also used. The chemical structures are shown in Scheme 1.

A mixture of standard compounds was prepared comprising 2.5 mg of **(1)** and **(2)** and 5 mg of **(3)** and **(4)** in 0.7 ml dmso-d_6 (Aldrich Chemicals, Gillingham, Dorset, UK). The test sample of fluticasone propionate comprised 20 mg dissolved in 0.7 ml dmso-d_6 and this contained a number of additional related components at varying levels, some of which were dimers and others monomers related to fluticasone propionate itself. Analysis by HPLC showed that the mixture contained 94.4% **(1)** by total area of UV absorption. Fluticasone propionate contains three fluorine atoms with the dimeric molecules containing four or more.

The ^{19}F NMR spectroscopic data were acquired at 376.50 MHz and at a temperature of 303 K using a Bruker DRX-400 NMR spectrometer equipped with a 5 mm, 4-nucleus, $^{1}H/^{19}F/^{13}C/^{31}P$ probe containing field-gradient coils and a Bruker BGU-10 gradient unit capable of delivering magnetic field gradient pulses along the magnetic field direction with strengths up to 590 mT m^{-1}. ^{19}F NMR spectra were obtained with ^{1}H decoupling ($^{19}F-\{^{1}H\}$ spectra) using the WALTZ method [9] summing 32 free induction decays into 64 K

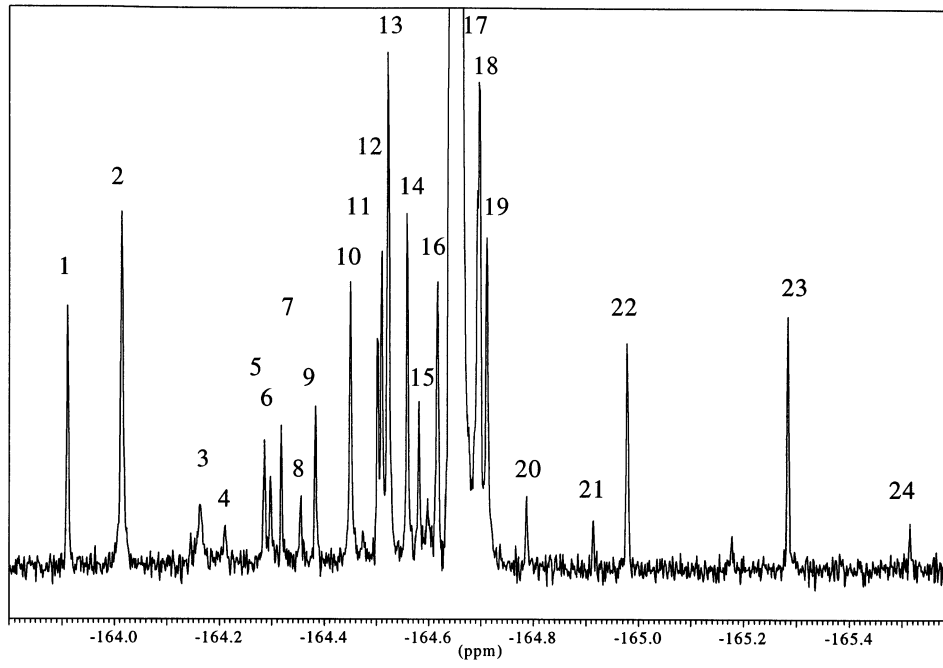

Fig. 1. Partial ^{19}F NMR spectrum of a batch of bulk fluticasone propionate **(1)** showing expansions of the region $\delta_F - 163.8 - \delta_F - 165.6$ where the peaks arise from F-9 in **(1)** and related molecules. Assignments are given in Table 1.

computer data points with a spectral width of 14880 Hz, an acquisition time of 2.20 s and a relaxation delay of 1.6 s. The data were resolution enhanced using the Lorentzian–Gaussian method and zero-filled by a factor of two prior to Fourier transformation. ^{19}F NMR chemical shifts were referenced to external $CFCl_3$ at δ_F 0 ppm.

Measurement of molecular diffusion coefficients was achieved using the LED method [4] for ^{19}F NMR observation modified by the inclusion of bipolar gradients [10] and ^{1}H decoupling using the WALTZ method [9]. The gradient strength was incremented from 10 to 41% in 32 increments of 1%. Other parameters were as above except that 896 transients were acquired into 32 K data points which were zero-filled by a factor of two before Fourier transformation. The diffusion period between the bipolar gradients (Δ) was 500 ms. The peak intensities were measured for each of the 32 values of the field gradient and the 24 most intense peaks in the ^{19}F NMR spectrum were used for the diffusion coefficient calculation. A diffusion coefficient was calculated for each ^{19}F NMR resonance using Eq. (1)

$$A_i = A_{i0} \exp[-D_i(2\gamma_F g\delta)^2 \times (\Delta + \tau/2 + 4\delta/3)] \quad (1)$$

where A_i is the intensity of resonance i at a gradient strength g, A_{i0} is the peak intensity at zero gradient strength, γ_F is the ^{19}F nuclear gyromagnetic ratio, δ is the pulse duration, τ is the time interval between each component of a bipolar gradient pair, Δ is the diffusion time and D_i is the diffusion coefficient. The diffusion coefficient corresponding to each ^{19}F NMR peak was derived by linear regression of ln(peak intensity) against g^2.

3. Results

Part of the $^{19}F-\{^{1}H\}$ NMR spectrum of the batch of fluticasone propionate in dmso-d_6 is given in Fig. 1 which shows an expansion of the region around $\delta_F - 164$. The peaks have been numbered and the chemical shifts are given in Table 1. The peaks in this region arise from F-9 of **(1)** and related compounds. In addition, there

Table 1
^{19}F NMR chemical shifts, mole% and diffusion coefficients of components of the partially purified batch of fluticasone propionate **(1)**[d]

Peak no.	Chemical shift (δ_F)	Mole%	Diffusion coefficient ($m^2\ s^{-1} \times 10^{-10}$)	Identity
1	−163.91	0.28	2.63	–
2	−164.01	0.47	1.93	Dimer[c]
3	−164.16	0.09	[a]	–
4	−164.21	<0.09	[a]	**(3)** Dimer
5	−164.29	0.19	[a]	**(5)** Monomer
6	−164.30	0.09	[a]	**(2)** Monomer
7	−164.32	0.19	[a]	–
8	−164.36	0.09	[a]	**(6)** Monomer
9	−164.38	0.219	2.44	**(7)** Monomer
10	−164.45	0.38	2.00	Dimer[c]
11	−164.50	0.28	2.69	–
12	−164.51[b]	0.38	2.54	**(1)** Monomer
13	−164.52	0.66	2.00	**(4)** Dimer
14	−164.56	0.47	2.35	Monomer[c]
15	−164.58	0.19	2.40	**(8)** Monomer
16	−164.62	0.38	2.31	Monomer[c]
17	−164.65	94.07	2.54	**(1)** Monomer
18	−164.70	0.56	2.48	Monomer[c]
19	−164.71	0.38	2.44	Monomer[c]
20	−164.78	0.09	[a]	–
21	−164.92	<0.09	[a]	–
22	−164.98	0.28	2.66	–
23	−165.28[b]	0.28	2.96	**(1)** Monomer
24	−165.51	<0.09	[a]	**(3)** Dimer

[a] Signal–noise ratio inadequate for diffusion measurement.
[b] ^{13}C satellites of **(1)**.
[c] Proposed on the basis of diffusion coefficient measurement.
[d] **(5)** as **(1)** but with OH and COOH substituted at C-17; **(6)** as **(1)** but with oxathiazole substituted at C-17; **(7)** as **(1)** but with COSH and COOEt substituted at C-17; **(8)** as **(1)** but with H and COOH substituted at C-17.

are a number of peaks around $\delta_F - 186$ and these arise from the corresponding F-6 nuclei (see Scheme 1). Also **(1)** itself has an additional resonance at $\delta_F - 191.98$ ppm arising from the CH_2F group. The ^{13}C satellite peaks of **(1)** have been identified at $\delta_F - 164.51$ and $\delta_F - 165.28$. Peaks heights have been measured on the 24 largest peaks in the region around $\delta_F - 164$ ppm and the mole% for each of these components has been calculated and these are also given in Table 1. There is good agreement for **(1)** itself between values obtained by ^{19}F NMR spectroscopy and HPLC with UV detection.

The batch of fluticasone propionate was also examined using HPLC and several components have been identified. This was achieved by addition of authentic materials synthesised in-house [11] and by the application of directly-coupled HPLC–NMR and HPLC–MS for the dimeric species [2]. Table 1 indicates the structure of these compounds, the mole% based on ^{19}F NMR peak integrals and whether the materials were monomeric or dimeric in nature from these analyses. There was good agreement between the results quoted here and those given earlier for the fluticasone-related dimers [2].

The proportions of the species detected using ^{19}F NMR spectroscopy are given as mole%. This is because some of the substances remain unidentified and therefore the molecular weight is not known. For monomeric impurities, differences in molecular weight will be small and hence relative proportions will not be greatly affected by conversion of mole% to weight%. For dimeric impurites,

the relative proportions will be approximately doubled if using weight%.

In order to investigate whether it was possible to discriminate by NMR which impurities were dimeric, diffusion coefficient measurement has also been used. To this end, a mixture of **(1)–(4)** was made up in dmso-d_6 solution and the diffusion coefficient corresponding to each resonance was measured using the bipolar LED NMR pulse sequence [10]. The diffusion coefficients of the standard compounds are given in Table 2 for each ^{19}F resonance. There was good consistency in values for different resonances in the same molecule and for monomers and dimers. Table 2 shows that the monomeric substances **(1)** and **(2)** had diffusion coefficients of $\approx 2.1 \times 10^{-10}$ m^2 s^{-1} whilst the dimers had values of ca. 1.6×10^{-10} m^2 s^{-1}. These diffusion data confirmed that the fluorine atoms from the monomeric species had a larger diffusion coefficient than the dimeric species and this was as expected since the smaller molecules would be expected to diffuse at a greater rate.

Diffusion coefficients were then measured for each ^{19}F NMR resonance arising from the bulk batch of **(1)**. The determined diffusion coefficients are also given in Table 1. The absolute values of the diffusion coefficients for each molecule differs somewhat from the values determined in the simple mixture of four compounds and this is probably due to differences in sample viscosity and/or temperature. Nevertheless, there is a clear distinction between the known monomer and dimer species.

Table 2
NMR-determined diffusion coefficients for **(1)–(4)**[a]

Identity	Chemical shift (δ_F)	Diffusion coefficients (m^2 $s^{-1} \times 10^{-10}$)
(3)	−164.22	1.61
(2)	−164.31	2.17
(4)	−164.52	1.55
(1)	−164.64	2.16
(3)	−165.53	1.61
(3)	−186.51	1.65
(2)	−186.55	2.16
(3) (4)	−186.71	1.58
(1)	−186.74	2.07

[a] Overlapped resonances at about $\delta_F - 192$ arise from **(1) (3)** and **(4)** and would therefore be expected to show complex multi-exponential intensity dependence on field gradient strength squared and thus an apparent diffusion coefficient was not determined for this resonance. **(1)** and **(2)** are monomeric and **(3)** and **(4)** are dimeric.

There is evidence from the diffusion coefficient data for the presence of three different dimers amongst the major impurity peaks in the ^{19}F NMR spectrum around $\delta_F - 164$. These are peaks 2, 10 and 13 in Table 1 of which peaks 2 and 10, which have not yet been assigned, are postulated as arising from dimers and peak 13 is from the dimer molecule **(4)**.

The results of the NMR study presented here on a partially purified production batch of fluticasone propionate serve to confirm that ^{19}F NMR spectroscopy of mixtures is a useful technique for characterising the number of components. If an internal standard is added and care is take to ensure complete T_1 relaxation between accumulation of successive FIDs, then the method can also provide good estimates of the relative molar proportions.

It is also shown that measurement of diffusion coeffients using the well-resolved resonances in a ^{19}F NMR spectrum of a mixture can be a useful initial technique for distinguishing the components according to their relative mobility and hence molecular size. Here this approach has been used to distinguish monomeric from dimeric impurity structures. In this case, the most accurate values will be determined for the main component fluticasone propionate itself as it represents about 94% of the total material but because of the lower signal–noise ratio of the NMR peaks from the impurity components the error on their peak intensity measurements will be increased. This has a consequence for the exponential fit to Eq. (1) and may result in a less precise estimate of the derived diffusion coefficients. It should be noted that this is usually greater than the fitting error to the curve of NMR signal intensity versus gradient strength squared used to derive the diffusion coefficients and represents a more realistic reproducibility error.

The diffusion coefficient measurement method should generally be of value in the analysis of

mixtures of products of synthetic organic chemistry such as in combinatorial chemistry. For example, it has been used to investigate which compounds in a mixture, such as from array or combinatorial synthesis, can bind to a protein receptor without separating the mixture of test compounds [12]. It has also been shown to be useful for the assignment of the components of other complex mixtures such as biofluids [6–8]. Therefore, it is potentially of general value in pharmaceutical and biochemical studies.

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