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Characterisation of impurities in bulk drug batches of fluticasone propionate using directly coupled HPLC–NMR spectroscopy and HPLC–MS

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Abstract

Directly coupled HPLC–NMR spectroscopic and HPLC–MS approaches have been used to confirm the identity of four known dimeric impurities in a partially purified batch of fluticasone propionate each at levels of 0.06–0.9% of parent compound based on UV absorption. It is also shown that HPLC–NMR spectroscopy of the main drug peak in the 'time-slice' mode of operation, in which the elution of the HPLC peak is sampled a short time intervals, can be used to investigate the purity profile of the single HPLC peak detected by UV absorption. These studies show that HPLC–NMR is of considerable value in rapidly assessing HPLC peak purity and hence will be of benefit in providing additional information to support submission for drug registration to regulatory agencies. © 1997 Elsevier Science B.V.

Keywords: Fluticasone propionate; Impurities; Spectroscopy; HPLC-NMR; HPLC-MS

1. Introduction

The manufacture and quality control of a drug and the licence for its sale are controlled by a variety of national regulatory authorities. As well as drug efficacy, there is a strong emphasis on the purity of final drug substances and it is necessary to obtain full characterisation and identification of any impurities at the level of $\geq 0.1\%$ of the UV peak area using HPLC analysis [1] to ensure understanding and optimisation of development chemistry and to monitor the adequacy of quality control during production. In order to characterise such impurities, currently it is necessary to isolate individual components by preparative HPLC. This work is often time consuming and expensive and yet may not give conclusive identification. Furthermore it is possible for the impurities to be degraded during sample extraction and

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purification. There is, therefore, a considerable need to develop and validate new methods for determining product purity and with this aim we have applied directly-coupled HPLC–NMR spectroscopic approaches to both the investigation of peak purity and for the characterisation of impurities, where additional data obtained from HPLC–MS, were also required.

The development and application of directly coupled HPLC–^{1}H NMR spectroscopy to the structural elucidation of drug metabolites has been described recently [2–5] where most studies used ^{1}H or ^{19}F NMR detection. HPLC–NMR spectroscopy can be carried out in 'continuous-flow', 'stop-flow' and 'time-slice' modes according to the analytical problem under study. The continuous-flow approach involves the rapid acquisition of a series of 1-dimensional NMR spectra while the eluent is flowing through the HPLC–NMR probe. This is most appropriate when the analyte concentration is high enough to enable an NMR spectrum to be collected in a few scans and is mainly limited to the sensitive NMR nuclei, ^{1}H and ^{19}F. Stop-flow HPLC–NMR is used where the retention times are known, where the analyte concentrations are lower or where multi-dimensional NMR experiments, with much longer data acquisition times, are required. The sample corresponding to an HPLC peak is stopped in the flow cell and then all required NMR data can be acquired. The third mode of operation is known as 'time-slicing' where the chromatographic elution is advanced in short time intervals of 10–30 s and NMR data are collected at each time point. This mode is used for the acquisition of several NMR spectra over a single chromatographic peak measured at various time points through the peak and can therefore be used to determine chromatographic peak purities [2–5]. We demonstrate here the application of HPLC–NMR spectroscopy to the characterisation of impurities arising during the chemical synthesis of a drug substance using as an example fluticasone propionate (**1**), a potent anti-inflammatory drug used for the treatment of the underlying inflammatory component of asthma. Several HPLC–NMR spectroscopic studies of chemical mixtures have been reported [6–9] but none of these were on drugs from production batches

2. Experimental

2.1. Materials

Deuterium oxide (Fluorochem Ltd, Glossop, U.K.), acetonitrile (ACN) Pestanal Grade (Riedel de Haan, Germany) and acetonitrile, HPLC grade (Merck, U.K.) were obtained commercially. Water was obtained from a Millipore water purification unit. The test samples of fluticasone propionate (**1**) were obtained from a partially purified batch of the drug substance, prior to formulation, from GlaxoWellcome, Montrose, UK. For the HPLC–NMR analysis 20 mg of sample was dissolved in 3 ml ACN/D_2O (2:1 v/v).

2.2. Analytical chromatography

The HPLC system comprised a Bruker LC22 pump and a variable wavelength UV detector (operated at 239 nm). The outlet of the UV detector was connected to the HPLC–NMR flow probe via an inert polyether(ether) ketone (PEEK) capillary. A column oven was used to enable separation at 40°C. The chromatography was controlled by a Bruker Chromstar HPLC data system (Bruker Spectrospin Ltd., Coventry, UK). A chromatography method was developed for baseline separation of the impurity peaks of interest. Analysis was performed on an Inertsil column (250×4.6 mm I.D.) packed with 5 mm Inertsil ODS-2. Typically 250 μl of sample was injected and a flow rate of 1 ml min^{-1} was used. Gradient elution was performed using D_2O (containing 0.05% trifluoroacetic acid) and acetonitrile (ACN), starting at 45:55 v/v ACN/D_2O increasing linearly to 60:40 v/v ACN/D_2O by 25 min and finally increasing to 75:25 v/v ACN/D_2O by 50 min.

2.3. NMR spectroscopy

The HPLC–NMR spectroscopic data were acquired using a Bruker AMX-600 NMR spectrometer equipped with a ^{1}H flow probe (cell of 4 mm I.D. with a volume of 120 μl). ^{1}H NMR spectra were obtained at 600.14 MHz in the stop-flow mode and by time-slicing. In order to suppress the

solvent signals, the ^{1}H NMR spectra were acquired using a pulse sequence based on the first increment of a 2-dimensional NOESY experiment (Bruker Spectrospin Ltd., Coventry, UK) with dual frequency irradiation for suppression of both the residual water and acetonitrile signals for 2 s before the first 90° pulse. Free induction decays (FIDs) were collected into 16K computer data points with a spectral width of 12 195 Hz and an acquisition time of 1.34 s. NMR spectra were acquired by accumulation of between 64 and 64K transients depending on the sample concentration. Prior to Fourier transformation, an exponential apodisation function was applied to the FID corresponding to a line broadening of 0.3 Hz. In time-slice mode, the HPLC elution was halted at 15 s intervals and ^{1}H NMR spectra were acquired using the same experimental parameters as above, accumulating typically 256 transients.

2.4. HPLC–MS

The HPLC–MS data was acquired on a Trio 1000 single quadrupole mass spectrometer (VG MassLab, Manchester, UK) equipped with a standard thermospray source operated in positive ion mode. Typically, the source was operated using a block heater at 200°C, an orifice nozzle temperature of 200°C and with the source ion repeller at 100 V. The spray chamber was pumped with an Edwards E1M18 rotary pump (Edwards High Vacuum, Crawley, UK) and the turbo pumps were backed by an Edwards E2M2 rotary pump. The mass spectrometer was scanned from 300–1000 amu in 0.8 s with a 0.1-s interscan delay. The solvent flow was supplied via a HP 1090Win Liquid Chromatograph (Hewlett Packard Ltd, Bracknell, UK) flowing at 1 ml min^{-1}. The chromatographic details were the same as used for HPLC–NMR spectroscopy except for substitution by H_2O for D_2O in the mobile phase.

3. Results

3.1. Chromatography

The gradient elution HPLC method described above was developed in order to separate four late eluting impurity peaks and the parent peak of fluticasone propionate (**1**).

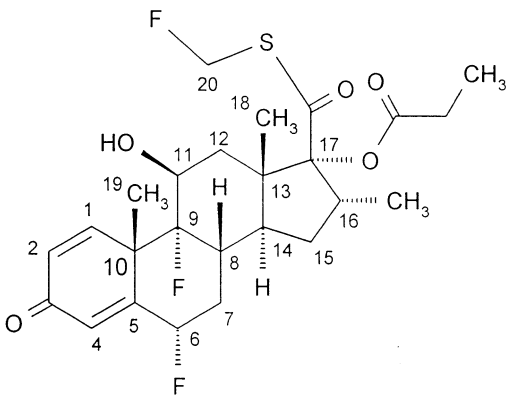


Fig. 1 shows the resultant chromatogram containing the peak of the parent compound (**1**) and the four impurity peaks of interest, labelled (**2**–**5**), eluting at retention times of 20, 26, 30 and 36 min, respectively. Other peaks present in the chromatogram are of various monomer impurities of (**1**) known to be by-products of its synthesis and identified by addition of authentic materials, preparative HPLC and NMR spectroscopy (unpublished work). Since structural identification was required for each of the four impurity peaks, it was necessary to achieve the highest possible concentrations of each compound in the NMR flow-cell. In order to obtain maximum NMR signal-to-noise ratios and to minimise the NMR acquisition time, high concentrations of the impurities were obtained by increasing the sample loading onto the column. The optimal injection volume was determined by running several experiments with increasing injection volumes and ensuring the peaks of interest did not broaden or coalesce. A 200-μl injection volume at a concentration of 6.7 mg ml^{-1} (**1**) in the mobile phase was used.

3.2. ^{1}H HPLC–NMR identification of the chromatographic peaks

Identification of the different peaks in the HPLC chromatogram was achieved by one di

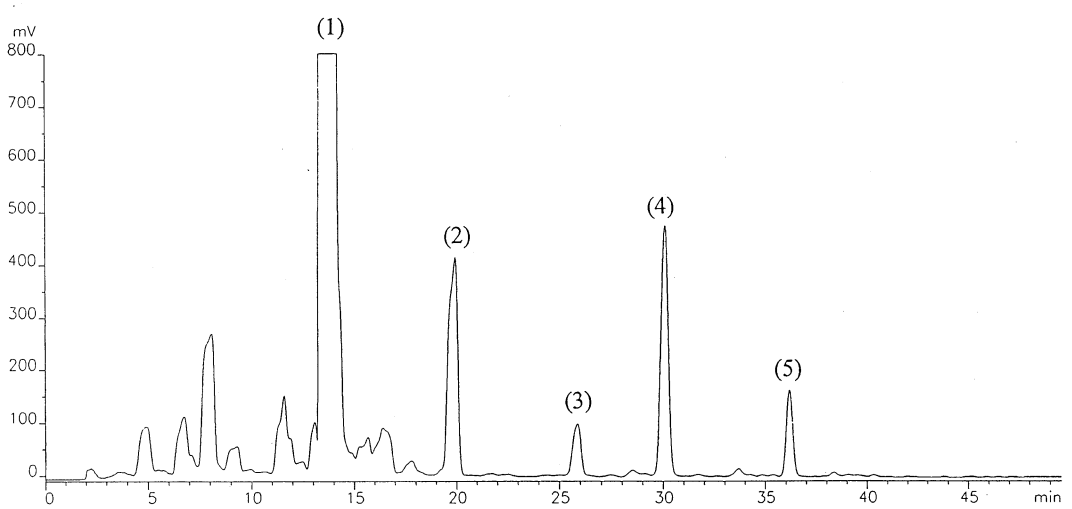
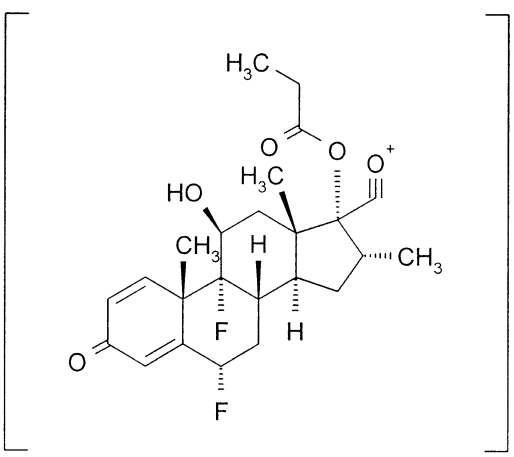


Fig. 1. HPLC separation with UV detection of fluticasone propionate. The parent compound is (**1**) and the four impurity components are (**2**–**5**).

mensional ^{1}H HPLC–NMR spectroscopy (as shown in Fig. 2) in the stop-flow mode and by HPLC-MS using positive ion thermospray ionisation (as shown in Fig. 3). The common fragment ion of 435, with the structure shown below,



also indicated that the steroidal substructure of fluticasone has remained intact. All the proposed structures for the four impurity peaks were consistent with the known chemical synthesis of fluticasone. Assignment of the NMR resonances and details of the mass spectral results which allowed structural identification of the four impurity peaks are summarised below.

*3.2.1. Fluticasone propionate (**1**)*

The ^{1}H NMR spectrum is shown in Fig. 2 (**1**) with some key assignments given in Table 1. The ^{1}H HPLC–NMR spectrum of this compound was fully assigned with the aid of a 2-dimensional COSY spectrum (data not shown) using standard methods. The purity of the HPLC UV peak for (**1**) was investigated by HPLC–NMR using the time-slice mode of operation. The elution was halted every 15 s over the peak and a ^{1}H NMR spectrum acquired. Typical results are seen in Fig. 4 starting near the leading edge of the UV peak (slice 4) where the signal-noise ratio is poor, through the main part of the peak (slice 6) where the signal-noise increases and finally to the trailing edge of the peak (slice 9) where the signal-noise is again lower. These spectra show no evidence for components other than fluticasone propionate in this HPLC peak.

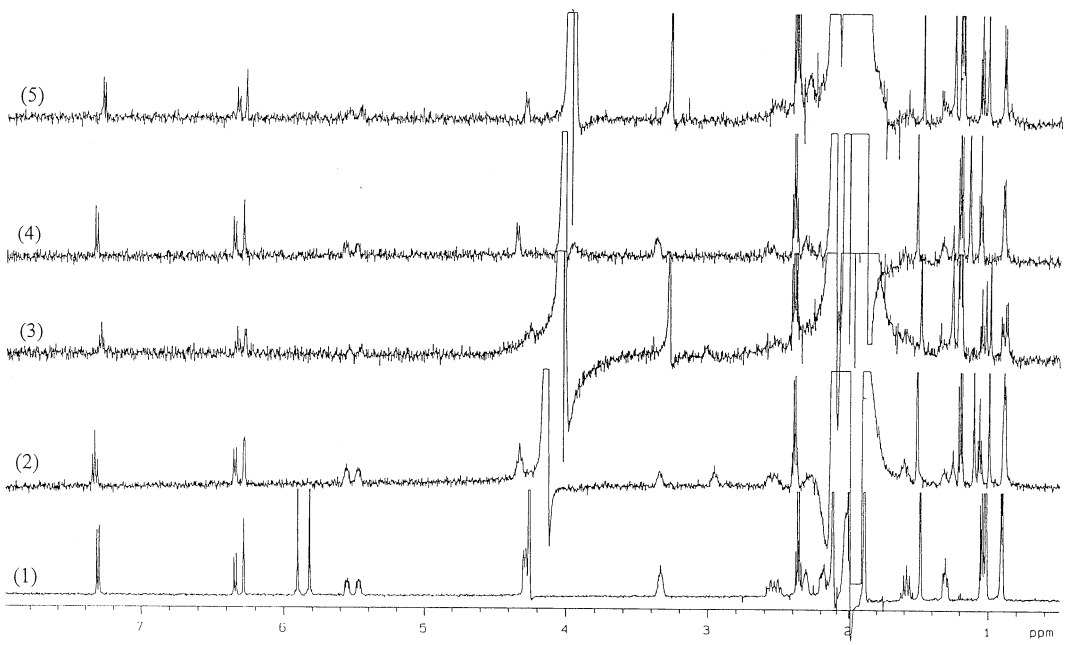
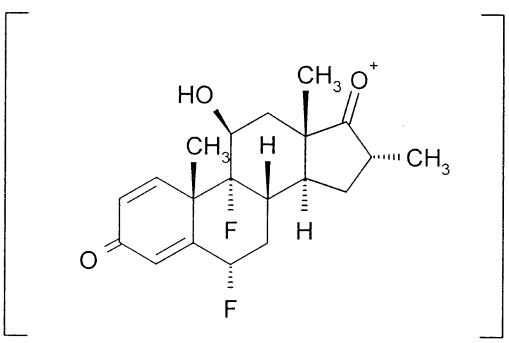


Fig. 2. Stop-flow 600 MHz ^{1}H NMR spectra from HPLC analysis of a production batch of fluticasone propionate. Fluticasone propionate (**1**); Compound (**2**), a disulphide-bridged dimer containing one propionate; Compound (**3**), a symmetrical disulphide-bridged dimer with C=S functionality; Compound (**4**), a symmetrical disulphide-bridged dipropionate dimer; and Compound (**5**), a disulphide-bridged dipropionate dimer with C=S functionality.

3.2.2. Compound **2**

It is clear that the NMR spectrum of this substance shown in Fig. 2 (**2**) is more complex than that for (**1**). Notably the doublet for H1 is now an apparent triplet as is the resonance for H11. In addition the doublet at around δ5.9 arising from the CH_2F group is no longer present. The doubling of the resonance for H1 and H11 and also for H16 where there is a larger chemical shift separation and the loss of the CH_2F group suggested an unsymmetrical dimer formed via the loss of the CH_2F group. The propionate group NMR signal intensity (triplet at δ1.06) indicates that only one propionate species is present. The HPLC–MS shown in Fig. 3 (**2**) gave a molecular ion $[M+H]^+ = 879$ which was consistent with a dimer and which was also consistent with a linkage across the thio-ester group which is present in the structure for fluticasone propionate. The base peak in the mass spectrum has $m/z = 351$ and this can be assigned the structure below.



All of the data are consistent with the relevant partial structure of compound (**2**) as shown below.

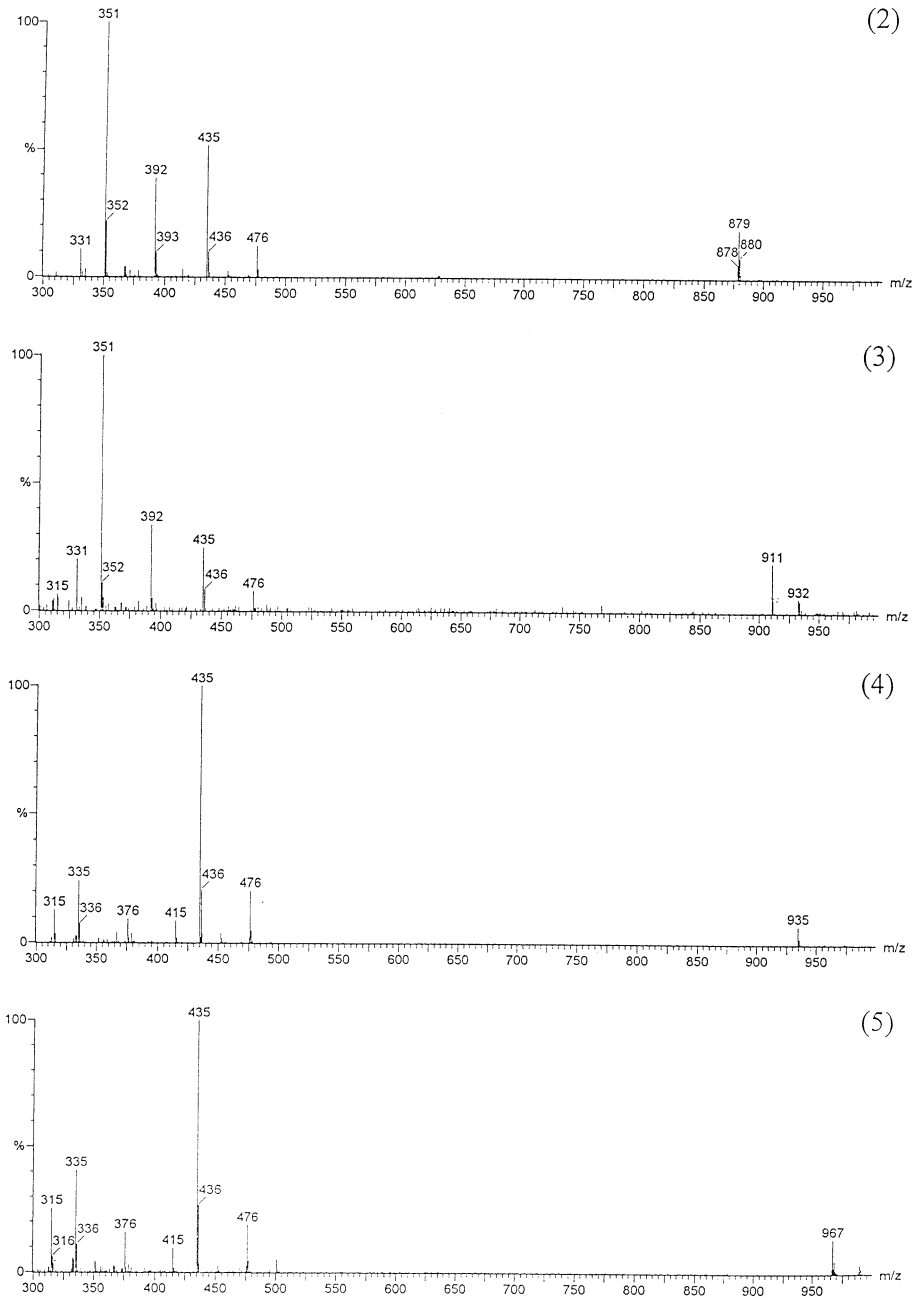
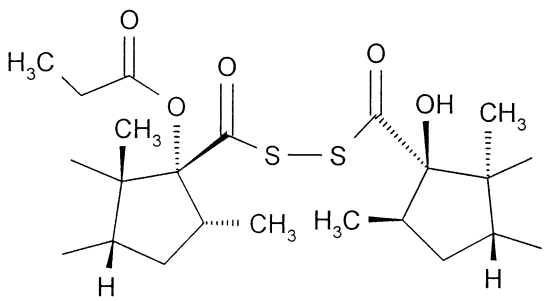


Fig. 3. HPLC-MS results for fluticasone propionate dimeric impurities. Compound (**2**), a disulphide-bridged dimer containing one propionate; Compound (**3**), a symmetrical disulphide-bridged dimer with C=S functionality; Compound (**4**), a symmetrical disulphide-bridged dipropionate dimer; and Compound (**5**), a disulphide-bridged dipropionate dimer with C=S functionality.

Table 1
Selected ^{1}H NMR spectroscopy and MS data for compounds (**1**–**5**)

	(1)	(2)	(3)	(4)	(5)
δ-H1 (*J*, Hz)	7.31 d(10.7)	7.34 d(10.7) 6.35 d (10.7)	7.28 d(10.7) 7.30 d(10.7)	7.33 d(9.8)	7.27 d(10.1)
δ-H2 (*J*, Hz)	6.34 d(10.7)	6.35 d(10.7)	6.32 d(10.7) 6.34 d(10.7)	6.35 d(9.8)	6.33 d(10.1)
δ(H11)	4.29 m	4.33 m	4.25 m	4.35 m	4.29 m
δ(H16)	3.33 m	3.34 m 2.96 m	3.27 m 3.01 m	3.36 m	3.31 m
δ(CH_2F)	5.87 d	—	—	—	—
Molecular ion (*m*/*z*)	501	879	911	935	967

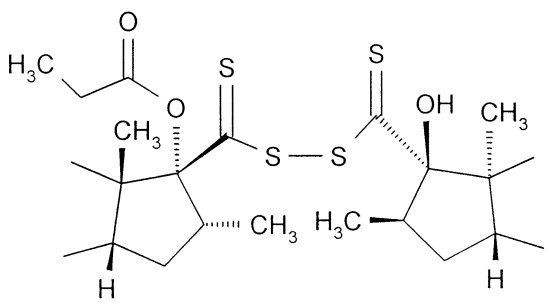
d, doublet; m, multiplet.



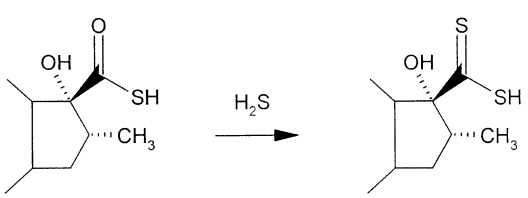
A second sample of drug substance from the same source was also studied. This contained only 0.06% by area of (**2**). The resulting NMR spectrum of (**2**) was identical to the original spectrum obtained (Fig. 2 (**2**)) but in order to obtain an adequate signal-to-noise ratio to enable structural identification it was necessary to signal average for 72 h. Thus it appears that HPLC–NMR at 600 MHz has a current effective detection limit for drug impurities of about this level.

3.2.3. Compound *3*

The ^{1}H NMR spectrum for this peak is shown in Fig. 2 (**3**). Again the spectrum is more complex than that for the parent drug. The doublets for H1, H2 and H11 are now apparent triplets. The doublet at around δ5.9 arising from the CH_2F group is no longer present. The doubling of the resonances for H1, H11 and also H16 where there is a large chemical shift separation and the loss of the CH_2F group suggested an unsymmetrical dimer formed via the loss of the CH_2F group. The propionate group signal (triplet at δ1.06) indicates that only one propionate species is present. HPLC–MS gave a molecular ion $[M+H]^+ = 911$ (Fig. 3 (**3**)) which also indicates a dimeric species and thus a partial structure for the compound (**3**) consistent with these data is shown below.



The fragment at $m/z = 435$ seen in the mass spectrum arises from this molecule by replacement of one of the sulphur atoms by oxygen. There is precedent for this type of reactivity from other sulphur-containing drugs such as azathioprine [10,11]. Compound (**3**) is synthetically feasible and is likely to be formed from a thioacid intermediate used in the synthesis which can react with hydrogen sulphide also present in the synthesis, thus



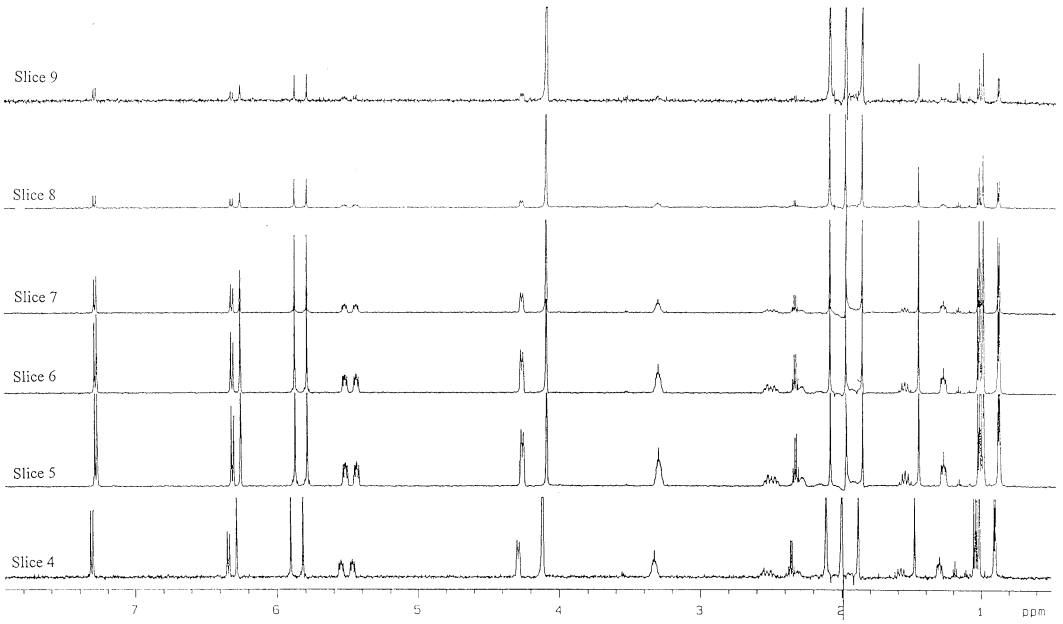
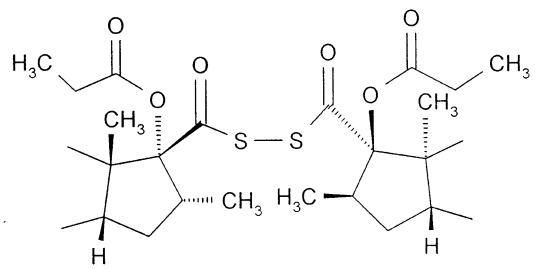


Fig. 4. Time-slice 600 MHz ^{1}H NMR spectra over the UV-detected HPLC peak corresponding fluticasone propionate.



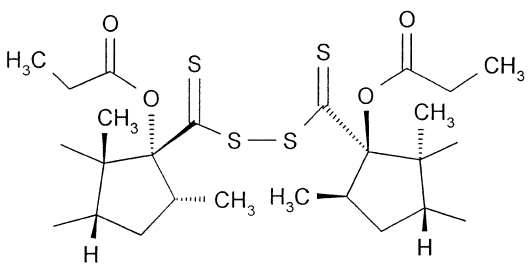
This conversion occurs at the monomeric stage and is followed by the dimerisation to give (**3**).

3.2.4. Compound **4**

The ^{1}H NMR spectrum for this peak is shown in Fig. 2 (**4**). The spectrum is very similar to that for the parent drug except that the doublet at δ5.9 arising from the CH_2F group is no longer present. No other significant changes were observed and HPLC–MS data, shown in Fig. 3(4) gave a molecular ion $[M+H]^+ = 935$. The NMR and MS data indicated a symmetric dimeric species and so the structure is consistent with (**4**) as shown below.

3.2.5. Compound **5**

The ^{1}H NMR spectrum for this peak is shown in Fig. 2 (**5**). The spectrum is again similar to that for the parent drug but with the doublet at δ5.9 arising from the CH_2F group no longer present. The HPLC–MS data shown in Fig. 3 (**5**) supports a dimeric species with $[M+H]^+ = 967$. The NMR spectrum indicated a species formed via the loss of the $-CH_2F$ group and a partial structure consistent with this information (**5**) is shown below. The incorporation of sulphur into the structure is consistent with the chemical synthetic route as outlined above for compound (**3**).



4. Discussion

NMR spectroscopy is universally recognised as being complementary to MS for structural elucidation of drugs and other small molecules. We have shown here for the first time that directly-coupled HPLC–NMR used in conjunction with HPLC–MS greatly enhances the ability to characterise impurities in a pharmaceutical product. As such, this approach is potentially significant as a general tool for purity analyses and would be expected to be important in speeding up production chemistry processes and for regulatory affairs. HPLC–NMR spectroscopy can detect and characterise impurities below the 0.1% peak level, the relevant limit for submissions to regulatory authorities.

NMR spectra on the peaks which were at a level of 0.2% or less of the parent drug required considerable and time consuming data acquisition to achieve acceptable signal-to-noise ratios. Although this appears to involve a high cost in NMR analysis time it would be justified in cases such as that found with fluticasone where there are no alternative analytical strategies or where alternative approaches have consistently failed. It may, therefore, be beneficial to concentrate the impurities before the HPLC–NMR by the application of solid phase extraction chromatography, column switching or concentration/enrichment of the impurities by preparative HPLC. Such techniques can be feasible because of the availability of large amounts of sample from drug production batches.

This work has focused on the evaluation of HPLC–NMR spectroscopy for characterising impurities in raw drug substances. However, the technique could be applicable to the investigation of formulated drug substances where impurities often appear as a result of the drug substance reacting with the formulation compound or on samples from degradation studies. These types of adduct can sometimes be unstable during sample extraction and purification. This area is of considerable practical importance in the pharmaceutical industry and is worthy of further study.

The application of HPLC–NMR together with HPLC–MS has led to the efficient identification of four novel impurities without recourse to development of time-consuming isolation and purification procedures. The isolation would have required laborious preparative HPLC using large amounts of sample and the final isolated product would also have been prone to contamination from solvent residues during preparation. The superior sensitivity of HPLC–NMR probes compared with normal detection has enabled the identification of these impurities using < 1 μg of each substance. This work has demonstrated the applicability of these techniques in the analyses of products of synthetic organic chemistry and is therefore potentially of great value in pharmaceutical analysis and is likely to be an important new approach for regulatory authorities.

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