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Fenofibrate raw materials: HPLC methods for assay and purity and an NMR method for purity

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Abstract

HPLC methods for drug content and HPLC and NMR methods for related compounds in fenofibrate raw materials were developed. The HPLC methods resolved 11 known and six unknown impurities from the drug. The HPLC system was comprised of a Waters Symmetry ODS column (100 × 4.6 mm, 3.5μm), a mobile phase consisting of acetonitrile–water–trifluoroacetic acid 700/300/1 (v/v/v) at a flow rate of 1 ml min^{-1}, and a UV detector set at 280 nm. Minimum quantifiable amounts were about 0.1% for three of the compounds and less than 0.05% for the other eight. Individual impurities in 14 raw materials ranged from trace levels to 0.25%, and total impurities from 0.04 to 0.53% (w/w). Six unknown impurities were detected by HPLC, all at levels below 0.1%, assuming the same relative response as fenofibrate. An NMR method for related compounds was also developed and it was suitable for 12 known and several unknown impurities. It requires an NMR of 400 MHz, or greater, field strength. Individual impurities in the raw materials analyzed ranged from trace levels to 0.24%, and total impurities from trace levels to 0.59%. Several lots contained small amounts of unknown impurities at trace levels. Three lots, all from the same manufacturer, contained an unknown impurity, not detectable by HPLC, which was not present in the other raw materials. It was estimated to be present at a level greater than 0.2%. The results for related compounds by the two techniques were consistent. The main differences stem from the low sensitivity of the HPLC method for some of the related compounds at 280 nm, or from the higher limits of quantitation by the NMR method for several other impurities using the conditions specified. A fifteenth raw material was not homogeneous in its content of impurity VI, a synthetic intermediate and possible degradation product. The HPLC/MS results provided information on the peak purity (number of components) for minor HPLC peaks, as well as structural data such as the molecular ions and diagnostic fragment ions. The HPLC/MS results showed that there were five unknown drug related impurities, for which there were no standards available. Results for the assay of 15 raw materials by HPLC were within the range 98.5–101.5%.

Keywords: Fenofibrate; Drug impurities; Assay; Related compounds; HPLC; NMR

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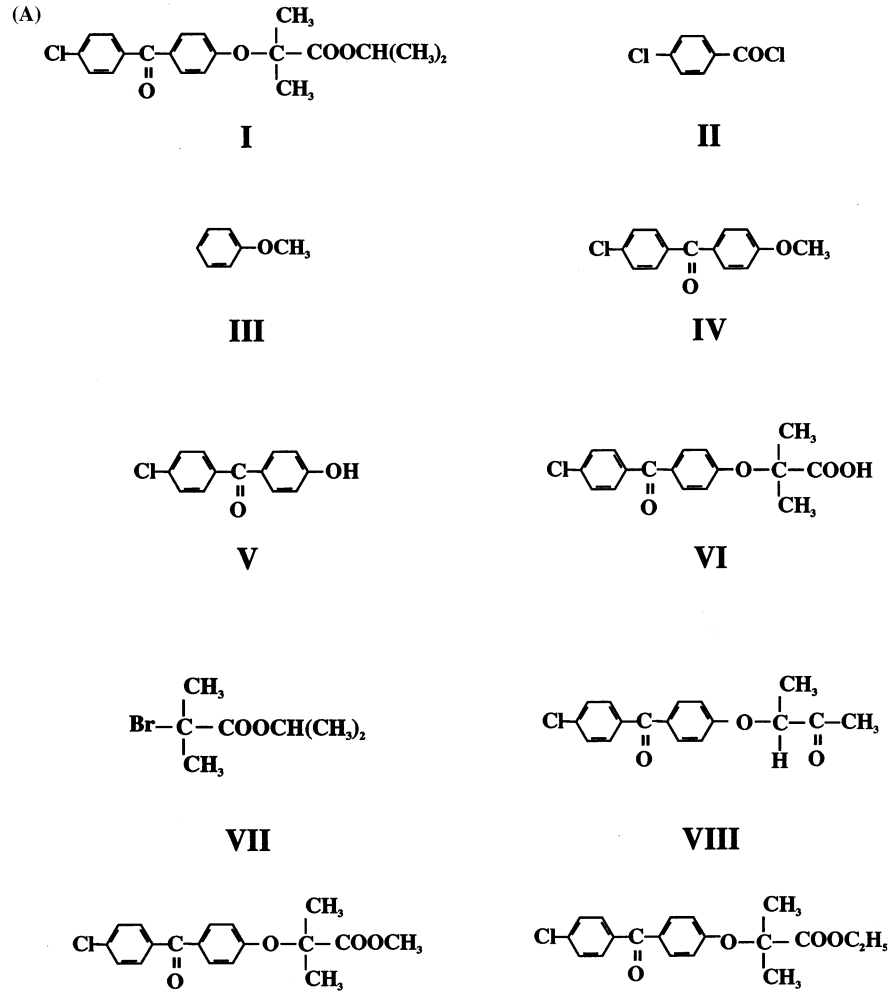

Fig. 1. Chemical structures of fenofibrate and related compounds.

1. Introduction

Fenofibrate is an antihyperlipidemic agent. Structures for fenofibrate (I) and several related compounds are provided in Fig. 1. Compounds I–XII and XVIII were available for method development. Compounds II–VII, and XVIII are starting materials or synthetic intermediates; VIII–XVI are by-products which occur in raw materials or are postulated on the basis of the synthesis route. Compound VI may be produced by hydrolytic deesterification of the drug under acidic or basic conditions, and compound V is a possible photodegradation product formed in solutions of acetonitrile–water (7/3) [1]. Compounds V and XVII–XXI are possible photodegradation products formed in methanolic solutions [2,3].

At the present time, there are no official standards for this drug in the USP, BP or EP. A proposed EP monograph, published in Pharmeuropa [4], while this work was under way, includes an HPLC method for drug assay and related substances. Impurities A, B and C (corresponding to compounds VI, V and XII, respectively) are listed in the transparency statement of this monograph and are required as standard reference materials. While the proposed EP

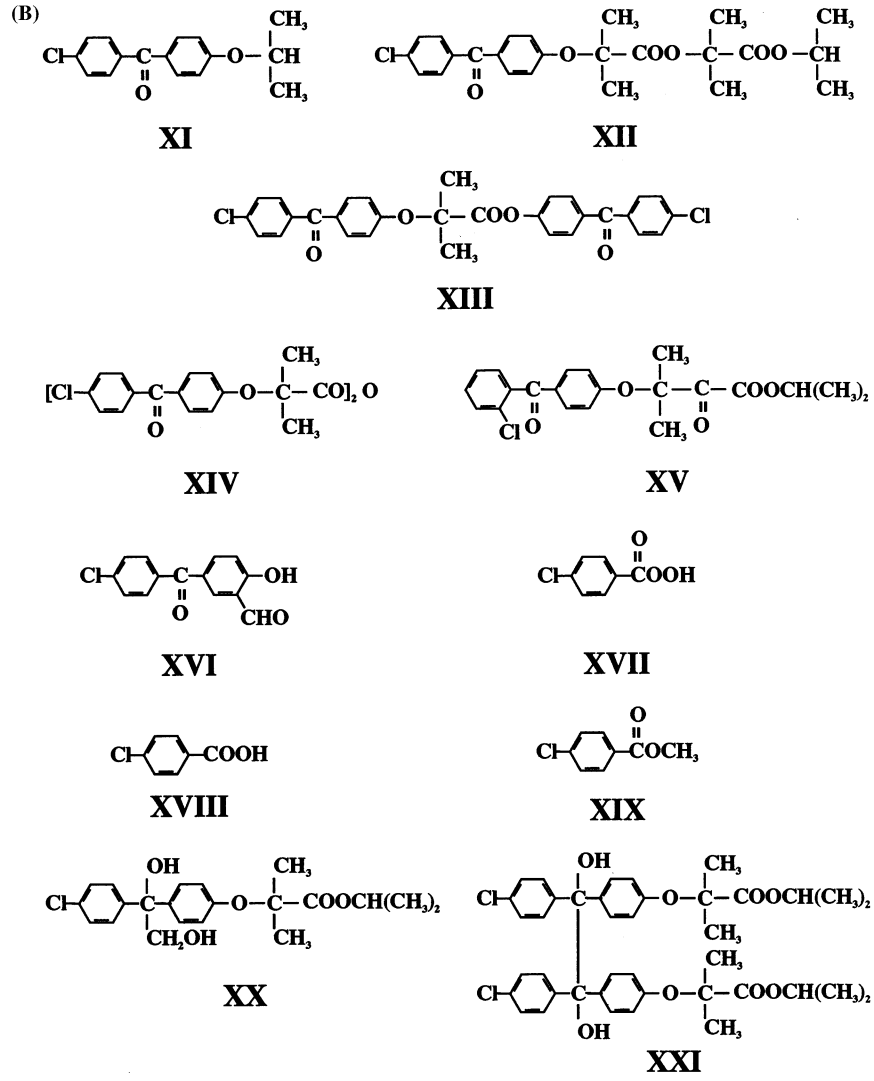

Fig. 1. (*Continued*)

method resolved all of the available related compounds from the drug using a base deactivated octylsilyl column, two pairs, III and VI, and X and XI were unresolved, and a third pair, II and V, was only partly resolved. Compound XVIII eluted with the solvent front.

The development of an HPLC method for the determination of drug content and impurities V and VI in fenofibrate raw materials was reported by Ji and Ye in 1987 [5]. Shoji et al. [1] reported TLC methods suitable for the detection of impurities V, VI and XII down to levels of 0.1%, an HPLC method for the assay of fenofibrate capsules capable of resolving the drug and impurities V, VI and X–XII with detection limits of 0.4 ng for each of these compounds, and a titration method for the assay of the bulk drug.

There have been several papers on the determination of fenofibrate or its metabolites in biological fluids using techniques such as HPLC [6,7], GC [8] or GC-MS [9,10].

Table 1
Chemical shifts of fenofibrate and known impurities

I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XVIII
7.745	8.074	7.339	7.810	7.771	7.771	5.063	7.797	7.748	7.744	7.788	7.753	8.046
7.738	8.052	7.320	7.787	7.766	7.749	5.047	7.791	7.726	7.722	7.766	7.731	8.024
7.733	7.510	7.317	7.720	7.754	7.726	5.031	7.779	7.718	7.714	7.720	7.719	7.465
7.721	7.488	7.298	7.699	7.749	7.705	1.917	7.774	7.697	7.693	7.699	7.698	7.443
7.716		6.976	7.464	7.718	7.465	1.293	7.717	7.460	7.458	7.461	7.459	
7.707		6.949	7.443	7.714	7.444	1.277	7.712	7.439	7.437	7.439	7.438	
7.702		6.947	6.982	7.702	6.974		7.700	6.865	6.877	6.945	6.948	
7.690		6.928	6.960	7.697	6.952		7.696	6.843	6.855	6.923	6.926	
7.686		6.926	3.896	7.466	1.708		7.466	3.772	4.265	4.683	5.081	
7.679		3.830		7.461			7.461	1.676	4.247	4.667	5.065	
7.459				7.449			7.449		4.229	4.652	5.049	
7.454				7.445			7.444		4.211	1.535	1.676	
7.449				6.926			6.928		1.672	1.394	1.528	
7.437				6.921			6.923		1.252	1.378	1.262	
7.432				6.909			6.911		1.234		1.246	
7.427				6.904			6.906		1.217			
6.884							4.735					
6.877							4.717					
6.872							2.201					
6.860							1.566					
6.855							1.549					
6.848												
5.132												
5.116												
5.100												
5.085												
5.069												
5.054												
5.038												
1.660												
1.210												
1.194												

Data is in ppm.

NMR is used extensively for the characterization of drug impurities and metabolites. In recent years, there have been several papers describing the quantitation of enantiomeric impurities in chiral drugs [11–15], and a few reports on the application of this technique to the quantitation of related compounds in drug raw materials [16–20].

This paper describes two independent methods for the determination of impurities in fenofibrate. These methods have been validated for a larger number of related compounds than the proposed EP method and require the use of only two reference standard materials. With only slight modifications of the HPLC method, HPLC/MS may be used to confirm or assist in establishing the identity of impurities.

2. Experimental

2.1. Instrumentation

The liquid chromatograph consisted of an HP 1090 M HPLC with a pump, an injector, an autosampler, a variable wavelength detector (HP 1050), a diode array detector (HP 1040) with optical unit upgrade), a computer (HP Vectra VL series 3, 5/90), a printer (HP Deskjet 500) and

Table 2
NMR response data for fenofibrate and related compounds

Compound	Spin rate (cps)	Chemical shift[a] (ppm)	Processing		Slope[d]	Intercept[e]	RSQ[f]	LOD[g] (%)	LOQ[h] (%)	R.S.D.[i]
			LB[b]	GB[c]						
II	10	8.05 (d) (avg)	0.343	0	0.30	0.01	0.998	0.016	0.033	1.2
III	47	3.81 (s)	0.343	0	2.01	0.02	0.996	0.003	0.006	2.8
IV	47	3.89 (s)	0.343	0	0.87	0.01	0.998	0.009	0.018	1.3
V	47	6.92 (d) (avg)	−0.5	0.343	0.22	0.00	0.0986	0.04	0.08	4.9
VI	47	1.69 (s)	−1	0.343	1.26	0.04	0.990	0.01	0.025	4.4
VII	10	1.91 (s)	0.343	0	2.18	0.02	0.998	0.002	0.004	0.9
VIII	47	2.20 (s)	0.343	0	0.77	0.00	0.998	0.011	0.022	1.8
IX	47	3.77 (s)	0.343	0	0.74	0.00	0.998	0.006	0.012	2.0
X	47	4.24 (q) (avg)	0.343	0	0.16	0.00	0.966	0.015	0.03	1.9
XI	47	1.39 (1/2d)	0.343	0	0.80	0.00	0.998	0.013	0.025	3.3
XII	10	1.53 (s)	0.343	0	0.81	0.02	0.998	0.004	0.008	4.3
XVIII	10	8.01 (d) (avg)	0.343	0	0.31	0.00	0.996	0.013	0.025	2.5

[a] The chemical shift of fenofibrate used for all quantitations was 1.66 ppm. (s), singlet; (d) (avg), doublet (average peak height); (1/2d), single peak of doublet; (q) (avg), average peak height of inner two peaks of a quartet.
[b] Line broadening.
[c] Gaussian broadening.
[d] Slope of the calibration curve for each related compound (relative response/% (w/w)).
[e] Intercept of the calibration curve.
[f] Square of the correlation coefficient (r^2).
[g] Limit of detection (% w/w) defined as $2 \times$ noise.
[h] Limit of quantitation (% w/w) defined as $4 \times$ noise.
[i] R.S.D. of the response of each impurity at the 0.2% level ($n = 5$).

software (HP HPLC 3D DOS Chemstation, version A2.02), all from Hewlett-Packard (Kirkland, P.Q.). The column was a Symmetry ODS 3.5 μm (100 × 4.6 mm) (Waters, Milford, MA).

The NMR spectrometer was a Bruker AM400 equipped with an Aspect 3000 computer and a 16 bit ADC (Bruker Spectrospin, Milton, Ont.). The spectra were acquired at spin rates of 10 and 47 cps. For each spectrum, a total of 256 scans with 32k data points were accumulated using a 5 mm proton probe.

The LC-MS system consisted of an HP 1090 HPLC with a diode array detector (Hewlett-Packard, Mississauga, Ont.) and a Micromass atmospheric pressure chemical ionization source (Quattro, Manchester, UK) at 650°C. The column was a Symmetry C-18 (2.1 × 150 mm) (Waters, Milford, MA). Using the same mobile phase as for the HPLC method at a flow rate of 300 μl min^{-1}, the separation was very similar to that described for the HPLC method, and the retention time of fenofibrate was 7.7 min.

2.2. Materials

Fenofibrate raw materials were obtained directly from five manufacturers. Compound III (reagent grade) was purchased from Sigma (St. Louis, MO), and compounds II, V and XVIII (all reagent grade) from Aldrich (Milwaukee, WI). Chemagis (Ramat-Hovav, Israel) provided samples of related compounds V–VII, IX and XII, Eprova (Schaffhausen, Switzerland) samples of V, VI, and VIII–XII, and NIHFI (Sofia, Bulgaria)

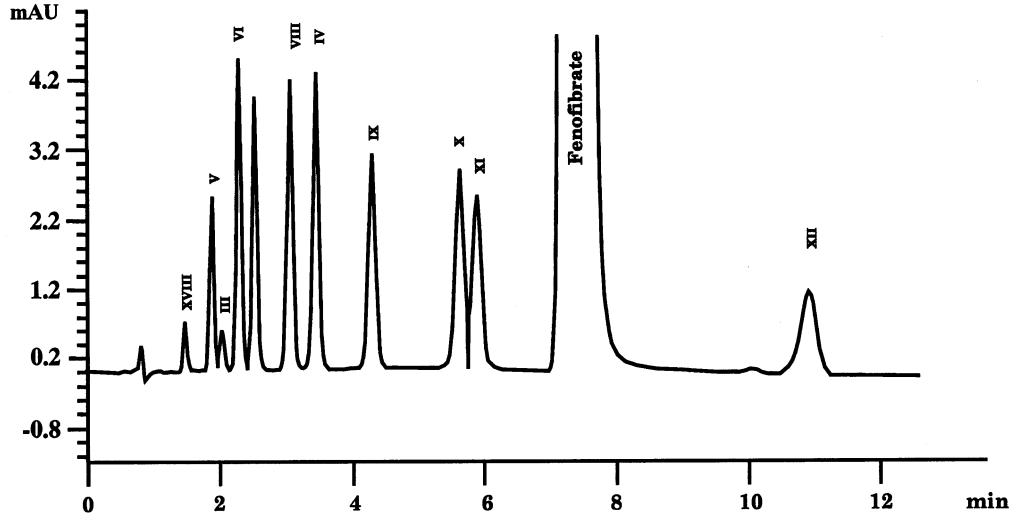

Fig. 2. Chromatogram of fenofibrate at 1 mg ml^{-1} and related compounds at about 0.002 mg ml^{-1}. (Note that compound II is not stable in solutions containing the drug and related compounds; it elutes on the tail of IV at about 3.6 min. Initially, compounds II and V appear to react to form a product with a retention time around 9.8 min, then later, a product with a retention time of 2.5 min (shown above unlabelled) is formed.)

samples of IV, VI, and IX–XI. The proton NMR and mass spectra of these compounds were consistent with their respective structures and they were of adequate purity to be used as reference compounds ($>90\%$, typically $>98\%$).

Acetonitrile and methanol (BDH, Toronto, Ont.) were of HPLC grade, and trifluoroacetic acid (Aldrich, Milwaukee, WI) was of spectrophotometric grade. The water used was distilled then deionized in a Barnstead Nanopure II system (Sybron/Barnstead, Boston, MA). $CDCl_3$ was 99.8 at.% D (Isotec, Miamisburg, OH).

2.3. Methods

2.3.1. HPLC

2.3.1.1. Mobile phase. The eluent consisted of acetonitrile–water–trifluoroacetic acid 700/300/1 (v/v/v) filtered through a 0.45 μm nylon filter (Lida, Kenosha, WI). The flow rate was 1 ml min^{-1}.

2.3.1.2. Solutions. Fenofibrate reference standard and raw materials were dried under pumping vacuum at 60°C for 2 h prior to use. The following solutions were prepared using acetonitrile and sonicated in an ultrasonic bath, when necessary, to dissolve the compounds: (1) a system suitability solution of 0.002 mg ml^{-1} (accurately known) a fenofibrate reference standard and 0.002 mg ml^{-1} 4′-chloro-4-hydroxybenzophenone (compound V), (2) a standard solution of 1 mg ml^{-1} (accurately known) fenofibrate reference standard, and (3) a test solution of 1 mg ml^{-1} (accurately known) fenofibrate raw material.

2.3.1.3. System Suitability. Six 5 μl aliquots of the system suitability solution were injected into the system. The system was deemed to be suitable if the efficiency of the column, calculated using the fenofibrate peak, was not less than 7000 plates, the resolution between compound V and fenofibrate was not less than 20, the retention time of fenofibrate was about 7.3 min, the relative retention time of compound V about 0.26, and the R.S.D. of the peak response from fenofibrate was not more than 5.0%. In addition for the assay of fenofibrate raw materials, six 5 μl aliquots of the standard solution were injected into the chromatograph. The R.S.D. of the peak response due to fenofibrate was required to be not more than 1.0%.

Table 3
HPLC response data for fenofibrate related compounds

Compound	RRT[a]	Slope (mAu·s ng^{-1})	Intercept (mAu·s)	R.S.Q.[b]	Relative response[c]	Est. LOD[d] (%)	Est. LOQ[e] (%)	R.S.D.[f] (%)
I	1.00	2.2215	1.11	0.9989	1.00	0.02	0.04	1.14
II	0.49	1.0220	−3.78	0.9989	0.46	0.07	0.14	0.58
III	0.28	0.2885	0.14	0.9990	0.13	0.05	0.1	0.68
IV	0.47	2.9112	−0.13	0.9999	1.31	0.006	0.013	0.43
V	0.26	2.7266	−0.04	0.9994	1.23	0.007	0.014	1.60
VI	0.31	2.4820	−0.44	1.0000	1.12	0.01	0.02	0.70
VIII	0.42	2.6723	0.14	1.0000	1.20	0.005	0.01	0.42
IX	0.58	2.5024	0.00	0.9999	1.13	0.01	0.02	0.63
X	0.76	2.3463	0.20	1.0000	1.06	0.007	0.014	0.69
XI	0.79	2.6759	−1.43	0.9916	1.20	0.015	0.03	0.42
XII	1.47	1.8374	0.06	1.0000	0.83	0.02	0.04	0.68
XVIII	0.20	0.1557	−0.04	0.9998	0.07	0.06	0.12	4.52

[a] Retention time relative to fenofibrate at about 7.3 min.
[b] The square of the correlation coefficient.
[c] Response relative to fenofibrate.
[d] Estimate of the limit of detection (i.e. 2 × noise), based on a 5 μg injection of fenofibrate.
[e] Estimate of the limit of quantitation (i.e. 4 × noise), based on a 5 μg injection of fenofibrate.
[f] R.S.D. for six injections at about the 0.2% level (i.e. 0.002 mg ml^{-1}).

2.3.1.4. Procedure. Aliquots (5 μl) of the system suitability solution, standard solution and test solution were injected separately into the chromatograph and run for 30 min. The percentage of each impurity in the raw material in the test solution was calculated using the formula

$$\%\text{impurity(i)} = 100(A_i/A_r)(C_r/C_u)$$

Table 4
Inter-day HPLC analysis of samples DD2 and EE2

RRT	Day 1	Day 2	Day 3	Mean	R.S.D.
Impurities in DD2 (%)					
0.25	0.01	0.01	0.01	0.01	0
0.76	0.01	0.02	0.02	0.02	34.6
1.47	0.08	0.09	0.10	0.09	11.1
Impurities in EE2 (%)					
0.31[a]	0.21	0.24	0.30	0.25	18.3
0.44	0.01	0.02	0.02	0.02	34.6
0.47	0.01	0.01	0.01	0.01	0
0.76	0.06	0.07	0.08	0.07	14.3
1.47	0.12	0.13	0.14	0.13	7.7

[a] Sample was not homogeneous in the content of this impurity (Table 5).

where A_i is the peak area due to the individual impurity, A_r is the area of the peak due to fenofibrate in the system suitability solution, C_r is the concentration of fenofibrate in the system suitability solution and C_u is the concentration of the raw material in the test solution. The percentage of fenofibrate in the test solution was calculated using the formula

$$\%\text{fenofibrate} = 100(A_u/A_s)(C_s/C_u)$$

where A_u and A_s are the areas of the peak due to fenofibrate in the test and standard solution, respectively, C_s is the concentration of fenofibrate in the standard solution and C_u is the concentration of the raw material in the test solution.

2.3.2. NMR

2.3.2.1. Solutions. The following solutions were prepared in separate 5 mm NMR tubes: (1) a standard solution of about 10.0 mg (accurately known) fenofibrate reference standard in 450 μl $CDCl_3$; (2) a system suitability solution of approximately 20 μg (accurately known from serial

Table 5
Variation of impurity VI content in sample EE2

	Weighing 1	Weighing 2	Weighing 3	Mean	R.S.D.
HPLC results (%)					
Day 1	0.23	0.21	0.20	0.21	7.2
Day 2	0.32	0.22	0.19	0.24	28.0
Day 3	0.36	0.26	0.28	0.30	17.6
NMR results (%)					
Day 1	0.25	0.52	0.46	0.41	34.6
Day 2	0.22	0.17	0.25	0.21	18.9
Day 3	0.22	0.19	0.26	0.22	15.7

dilution) compound V and 10.0 mg (accurately known) fenofibrate reference standard in 450 μl $CDCl_3$; and (3) a test solution of about 10.0 mg (accurately known) fenofibrate in 450 μl $CDCl_3$.

2.3.2.2. System suitability. The purity of the $CDCl_3$ used to prepare the solution was verified by obtaining a spectrum of the solvent under the same conditions as the samples and verifying that the only peaks present were those for $CHCl_3$ (7.26 ppm) and H_2O (1.58 ppm).

Spectra for the standard solution and the system suitability solution were acquired at a spin rate of 47 cps and data were processed as described below. The spectrum of the system suitability solution was re-acquired after 1–2 h. The system was deemed suitable if:

1. The resonance at 6.92 for compound V was resolved from the drug resonances (See Table 1) and had a signal to noise ratio of at least 4:1.
2. When the two spectra for the system suitability solution were compared, the intensity of the peak for compound V did not change relative to that for fenofibrate over time.

2.3.2.3. Data processing. The parameter settings provided in Table 2 were used. Zero filling to 64k was applied and the appropriate window function (line broadening, then if impurities V or VI were present, re-processing using the appropriate line broadening and gaussian broadening functions), Fourier transformation, phasing and baseline correction were performed.

2.3.2.4. Procedure. A proton spectrum of the test solution at each of the spin rates (10 and 47 cps) was acquired and the data processed as described above in Section 2.3.2.3.

The intensity of resonances corresponding to impurities at the chemical shifts indicated in Table 2 were determined and the amount of each individual impurity calculated using the formula:

$$Q = \frac{(h_i/h_d) - y_i}{m_i}$$

where Q_i is the amount of each individual impurity in the drug sample as a percentage of the total amount of drug (w/w), h_i is the intensity of the impurity resonance specified in Table 2, h_d is the intensity of the drug resonance at 1.66 ppm, y_i is the y intercept for the calibration curve for the particular impurity (Table 2), and m_i is the slope of the calibration curve for the particular impurity (Table 2)

The use of this equation takes into account the number of protons and multiplicity of the resonances. The chemical shifts provided in Table 2 are those which give the best response for each impurity and are free from interference from the other known impurities. Other resonances for each impurity are listed in Table 1.

To estimate the level of impurity V, it would be necessary to subtract the contributions from impurities VI, VIII, XI and/or XII, if present, from the signal at 6.92 ppm. Since impurity V was not found in any of the samples, the reproducibility of its quantitation was not evaluated.

Table 6
System suitability test results

Date (YY/MM/DD)	Retention time (min)		Efficiency (plates/column)	Resolution	R.S.D. (%) system suitability solution	R.S.D. (%) assay standard solution
	Compound V	Fenofibrate				
Lab 1—column no. T52342						
960527	1.883	7.429	12638	28.06	3.37	0.42
960603	1.891	7.473	12087	27.44	0.54	0.17
960606	1.880	7.428	11307	26.54	2.20	0.10
960718	1.870	7.318	7544	23.95	0.79	0.30
960719	1.861	7.228	7236	23.36	0.68	0.12
960821[a]	1.918	7.457	9335	24.12	0.86	0.13
Lab 2—column no. T60951 01						
	2.008	7.312	9615	22.2	1.78	0.31
Lab 3—column no. T60961 02						
	1.95	7.289	9197	24.55	2.09	0.27

[a] A different detector of the same make and model but with a larger dead volume was used.

Table 7
Results of HPLC assay of fenofibrate raw materials[a]

Raw material code	First weighing	Second weighing	Third weighing
CA1	99.7	99.9	
CB1	99.5	100.9	
CC1	99.4	99.8	
CC2	100.0	100.0	
CC3	100.1	100.1	
CC4	100.0	99.8	
CC5	100.0	100.1	
DD1	100.1	101.1	
DD2			
Day 1	99.5	100.2	99.9
Day 2	99.1	99.6	101.2
Day 3	98.7	98.9	99.9
DD3	100.4	99.9	
ED1	100.7	100.4	
ED2	100.4	100.2	
EE1	100.5	99.5	
EE2			
Day 1	99.4	98.8	99.2
Day 2	99.0	100.1	100.2
Day 3	99.2	100.0	100.0
EE3	99.8	100.3	

Data is in percent.
[a] Samples were assayed using DD1 as standard. Each value is the average of two injections.

Resonances arising from unknown impurities were identified by referring to Table 1 which contains a list of all the chemical shifts for fenofibrate and those for the major peaks for each multiplet for each of the available known impurities. The spectrum of the test solution at each spin rate was examined to determine if there were any resonances which did not correspond to the drug peaks, their spinning-side-bands or their carbon-13 satellites or to any of the impurities previously quantified. Any unassigned resonances were then assumed to arise from one or more unknown impurity(ies). Examination of the multiplicity and intensity of the peaks, compared to known impurities or the drug, could be used to give an estimate of the level of unknowns. The chemical shifts listed in Table 1 are for pure compounds and thus may differ slightly from those found for the compound at lower levels in solution with 10 mg of drug.

3. Results and discussion

3.1. HPLC

3.1.1. Specificity

All of the available related compounds were well resolved from the drug (Fig. 2). Compounds VII and VIII were unresolved from each other, and three other pairs, V and III, IV and II, and X and XI were only partially resolved. The HPLC method was not suitable for the determination of compound VII because it was not resolved from compound VIII. Since compound VII showed virtually no absorption at 280 nm, it did not interfere with the quantitation of VIII. Because compound VII was readily quantitated by NMR, no attempt was made to modify the HPLC method. A number of unknown compounds present in the samples of raw materials were also resolved from the drug (RRT = 0.44, 0.73, 1.21, 1.32, 1.44).

Table 8
Impurities in fenofibrate by HPLC

RRT[a]	0.23	0.26	0.31	0.42	0.44	0.47	0.58	0.73	0.76	0.79	1.21	1.32	1.44	1.47	Total impurities
Impurity[b]	Unknown	V	VI	VIII	Unknown	IV	IX	Unknown	X	XI	Unknown	Unknown	Unknown	XII	
Raw material code															
CA1		Tr[c]					0.03		0.01	0.01	0.01			0.16	0.22
CB1		tr				tr	tr		0.02	0.01				0.14	0.17
CC1		tr							0.01	0.01		tr		0.11	0.13
CC2		0.01							0.01	0.01				0.13	0.16
CC3		tr							0.02					0.12	0.14
CC4		tr							0.02					0.12	0.14
CC5		tr							0.03					0.10	0.13
DD1									0.01				0.02	0.01	0.04
DD2		0.01	tr				tr	tr	0.02	tr				0.09	0.12
DD3		tr	0.01	0.01			0.03		tr					0.02	0.07
ED1	0.01	0.01	0.01				0.03		0.01					0.03	0.10
ED2	0.01	tr	tr				0.02		0.01					0.03	0.07
EE1		tr	0.11		0.04	0.01	0.02		0.09	0.01				0.25	0.53
EE2			var[d]		0.02	0.01			0.07					0.13	var
EE3		tr	0.03		0.01	0.01			0.08	tr				0.14	0.27

Data is in % (w/w).
[a] Retention time of impurity peak relative to fenofibrate at about 7.3 min.
[b] Identity of impurity, based on retention time only.
[c] Trace (peak was detected but could not be integrated).
[d] Variable (sample was not homogeneous; levels of VI varied from 0.19 to 0.36%).

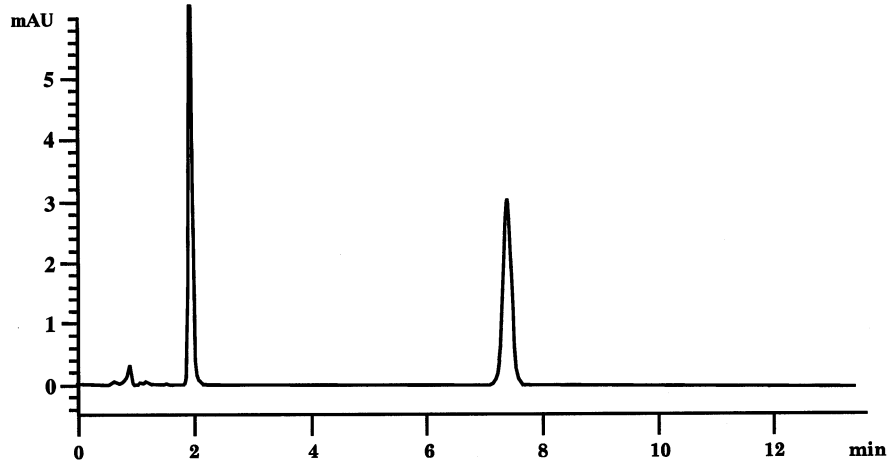

Fig. 3. Chromatogram of the system suitability solution.

Compound II was not stable in solutions containing mixtures of the drug and related compounds; it eluted on the tail of IV at about 3.6 min. Initially, a product with a retention time of about 9.8 min was formed, then later, a product with a retention time of 2.5 min.

3.1.2. Linearity, sensitivity and precision

The linearity of response of the HPLC system to fenofibrate related compounds was verified. This was done by preparing two stock solutions, making 5 or 6 dilutions from each stock over the range of 0.05–2.0%, determining the response of the system to these dilutions and analyzing the data by linear regression. The slope, intercept, square of the correlation coefficient, and response relative to fenofibrate are presented in Table 3 for each related compound.

The response of fenofibrate at concentrations ranging from 50 to 200% of the assay concentration (0.5–2.0 mg ml^{-1}) was also linear ($r^2 = 0.9999$, slope = 2.198 mAU·s ng^{-1}, and intercept = 202 mAU·s).

The precision of the system for related compounds was determined by calculating the R.S.D. of the peak responses ($n = 6$) for each compound at the 0.2% level (Table 3). Generally the R.S.D.s were below 5.0% at the proposed limit of 0.2%.

The precision of the method for related compounds was determined by calculating the R.S.D. of the average peak responses for each impurity on three different days for samples DD2 and EE2. The results are provided in Table 4. Note that the levels of VI in EE2 were variable (Table 5). The HPLC results for this sample ranged from 0.19 to 0.36% and the NMR results from 0.19 to 0.52% for the level of this impurity.

The precision of the system for assay was determined as part of the system suitability test (Table 6). The R.S.D. values for six consecutive injections of the assay standard solution were below 0.58% which is considered acceptable for a method where the analysis is to be conducted in duplicate and the acceptance range of the assay is 98.5–101.5% [21].

The inter-day precision of the method for assay was determined by analyzing sample DD2 in triplicate on three days (Table 7). The mean of the average assay value for each of these three days was 99.7% (R.S.D. = 0.45%).

3.1.3. Limits of detection and quantitation

The limits of detection (LOD) and quantitation (LOQ) provided in Table 3 were estimated by measuring the noise produced by a blank and the signal to noise ratio of a sample at the 0.05% level, and estimating the concentration of the related compound that would provide a signal to noise level of 2 (LOD) or 4 (LOQ).

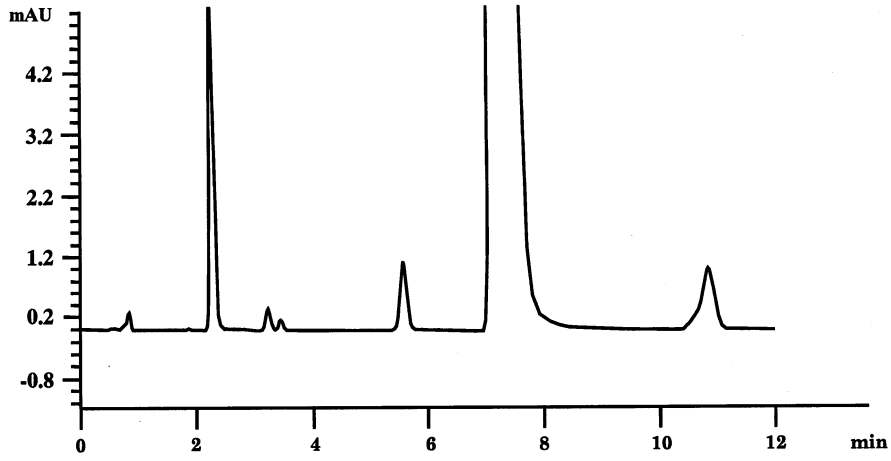

Fig. 4. HPLC chromatogram of a test solution of sample EE2.

3.1.4. Stability of solutions and robustness

The test solutions of fenofibrate raw materials showed no increase in the amount of impurities over a 24 h period.

TFA in the mobile phase was required for the elution of impurities II, VI and XVIII within a reasonable period of time. Increasing the concentration of acetonitrile by 25 ml (i.e. 725/275/1 (v/v/v) acetonitrile–water–trifluoroacetic acid) reduced the retention time of the drug to 6.3 min, and conversely an increase in the concentration in water to 675/325/1 (v/v/v) increased the retention time of the drug to 8.8 min. In each case the separation of the compounds remained adequate. A small change in the concentration of trifluoroacetic acid in the mobile phase (i.e. 700/300/0.5 or 700/300/1.5 (v/v/v) acetonitrile–water–trifluoroacetic acid) had virtually no effect on the separation.

The separation was reproducible on several columns containing different lots of the same type of packing material from the same manufacturer.

A system suitability test was devised for this method and the parameters were monitored during the method development and sample analysis. These data, presented in Table 6 were used to set the system suitability criteria of the method. The compounds chosen for the system suitability solution were fenofibrate and compound V which is a commercially available precursor of the drug. Fenofibrate in the system suitability solution serves as an external standard for the quantitation of the impurities in the test solutions (Fig. 3).

3.1.5. Analysis of available products

The results for the assay of 15 raw materials by HPLC are summarized in Table 7. All the samples would meet the proposed requirements of 98.5–101.5%.

The results for the determination of impurities in these samples by HPLC are presented in Table 8. Individual impurities ranged from trace levels to 0.36%, and total impurities from 0.04 to 0.59%. Six unknown impurities were detected by HPLC, all at levels below 0.1%, assuming the same response as fenofibrate. One of the unknown impurities eluted immediately before impurity XII. All the samples, except CA1, exhibited either a broad peak for XII or fronting of the impurity XII peak which could indicate the presence of this unknown in the raw material at a lower concentration than XII (assuming the same sensitivity as the drug) (Fig. 4). In sample DD1 the level of the unknown was higher than XII and two partially resolved peaks could be observed.

3.1.6. Ruggedness

Three samples were sent to two external laboratories for analysis using HPLC methods. These laboratories used different HPLC systems but the

same brand of HPLC column as the original laboratory. The system suitability requirements were easily met (Table 6). Results for the determination of drug content and related compounds in three lots of raw material were consistent with those of the original laboratory and are summarized in Table 9.

3.2. NMR

3.2.1. Specificity

Each of the available related compounds had a resonance which was sufficiently well separated from those of the drug and other related compounds to allow quantitation (Tables 1 and 2 and Fig. 5). For compounds V and VI, it was necessary to use some resolution enhancement (line broadenings of -0.5 and -1.0, respectively). The resonances which are used for quantitation of impurity V, at about 6.92 ppm, overlap with the resonances from other impurities (II, VII, XII and XVIII). These other impurities were quantified using other resonances. It is therefore possible in a complex mixture to calculate the levels of these impurities, then subtract the corresponding estimated total intensity these impurities would have at 6.92 ppm from the measured intensity to estimate the level of V.

For some of the impurities, the peaks used for quantitation were relatively close to those of the drug. This sometimes led to interference from the spinning-side-bands from the drug resonances. No convenient single spin rate could be found for which there was no interference for all the impurities. Thus, it was necessary to acquire the spectra at two different spin rates (47 and 10 cps) to alleviate the spinning-side-band problem.

Table 9
Comparison of HPLC results from different laboratories

Impurity RRT	Lab 1[a]	Lab 2	Lab 3	Mean
Lot DD1				
Assay	100.6	99.9	100.3	100.3
0.27			tr	
0.32			tr	
0.59			tr	
0.76	0.01	0.01	0.01	0.01
1.44	0.02	0.03	0.03	0.03
1.47	0.01			
Lot DD3				
Assay	100.2	99.6	99.3	99.7
0.26	tr		tr	
0.32	0.01	tr	0.01	
0.44	0.01	0.01	0.01	0.01
0.59	0.03	0.03	0.03	0.03
0.76	tr		tr	
1.46	0.02		0.02	
Lot EE1				
Assay	100.0	99.0	99.7	99.6
0.26	tr		tr	
0.31	0.11	0.10	0.10	0.10
0.45	0.04	0.03	0.04	0.04
0.48	0.01	tr	0.01	
0.59	0.02	0.02	0.02	0.02
0.76	0.09	0.08	0.09	0.09
0.79	tr			
1.46	0.25	0.22	0.23	0.23

Data is in percent.
[a] Lab 1 was the laboratory that developed the method.

3.2.2. Linearity, sensitivity and precision

The sensitivity of the NMR system to fenofibrate impurities was determined in the following manner. Duplicate stock solutions containing about 1 mg ml^{-1} (accurately known) of each impurity in $CDCl_3$ were prepared. Fenofibrate samples (10.0 mg), free of the impurity to be assayed, were accurately weighed into 5 mm NMR tubes. These tubes were spiked with aliquots of the stock impurity solution to give levels of 0.05–1.0% (w/w) (except for impurity V, where the range was 0.1–1.0% (w/w) because the signal to noise level at 0.05% was too low for quantitation). The volume in the tube was made up to 450 μl with $CDCl_3$. Six different concentrations from each stock solution were prepared. After processing as described above, the normalized impurity intensity at the specified resonance (h_n) was obtained using the formula:

$$h_n = 100 \cdot (h_i/(h_i + h_d))$$

where h_i is the intensity of the impurity at the

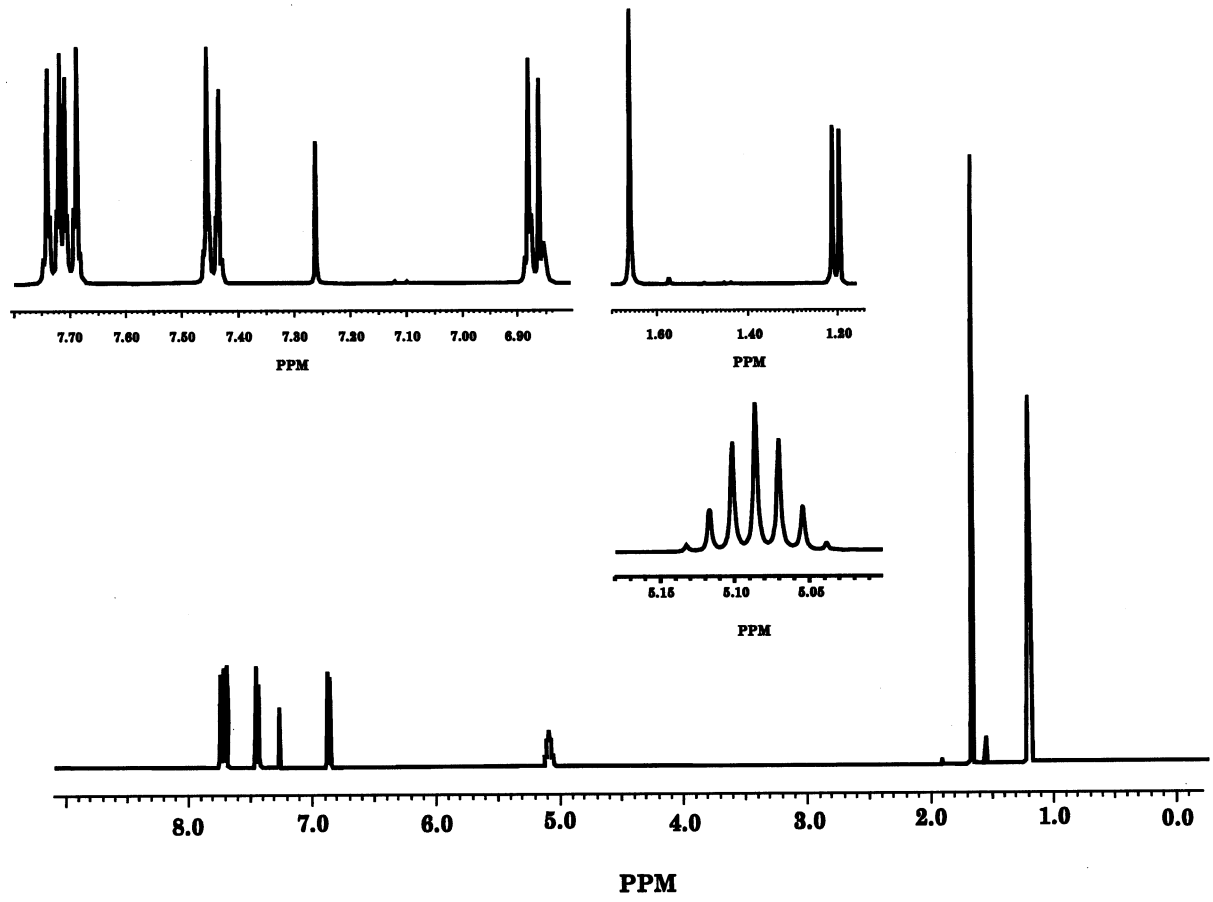

Fig. 5. NMR spectrum of fenofibrate raw material DD3.

specified resonance, and h_d is the intensity of the drug resonance at 1.66 ppm

The normalized impurity intensity was plotted against the known weight percent of the impurity. The slope, intercept and correlation coefficient for each impurity are provided in Table 2.

The precision of the system for each related compound was determined by preparing five 10 mg samples of drug spiked with an appropriate amount of a stock solution of the related compound to give a concentration of 0.2%. The R.S.D. of the impurity intensity responses was obtained and the results are shown in Table 2.

The precision of the method was determined by analyzing samples DD2 and EE2 in triplicate on three different days. The mean of the average impurity level on each day was: 0.08% (R.S.D. = 2.8%) for impurity XII in sample DD2; and 0.12% (R.S.D. = 1.3%), 0.09% (R.S.D. = 3.6%) for impurities XII and X, respectively in sample EE2. Sample EE2 also contained trace levels of impurity IV and a variable content of impurity VI (Table 5).

3.2.3. Limits of detection and quantitation

Although the low end for the calibration curves was 0.05%, signal to noise ratios at this level indicated that it should be possible, in most cases to quantify the impurities at much lower levels. The limits of detection (2 × noise) and quantitation (4 × noise) were estimated for each of the available related compounds and are included in Table 2.

Table 10
Impurities in fenofibrate by NMR

Impurity	IV	VI	VII	IX	X	XII	Unknown	Total impurities
Raw material code								
CA1			tr[a]	0.02		0.17		0.19
CB1						0.12		0.12
CC1						0.08		0.08
CC2						0.11		0.11
CC3						0.11		0.11
CC4						0.10		0.10
CC5						0.16		0.16
DD1						tr		tr
DD2						0.08		0.08
DD3				0.03				0.03
ED1				0.02		tr		0.02
ED2			tr	0.02		tr		0.02
EE1	tr	0.06		tr	0.09	0.24	>0.2[b]	>0.59
EE2	tr	var[c]			0.09	0.11	>0.2[b]	var
EE3	tr	tr			0.09	0.11	>0.2[b]	>0.40

Data is in % (w/w).
[a] Trace (below the LOQ).
[b] Each of these three samples contains at least one unknown with a triplet at 4.13 ppm, a quartet at 3.72 ppm and a triplet at 0.85 ppm. Levels of the unknown were estimated by comparison of peak heights to the resonance due to the ethoxy CH_2 in impurity X which was also present in these samples.
[c] Variable (sample was not homogeneous in the content impurity VI; levels ranged from 0.19 to 0.52%).

3.2.4. Stability of solutions and robustness

One bottle of $CDCl_3$ contained an impurity, possibly HCl, which caused impurity V to decompose. Thus it is necessary to confirm that the NMR solvent is pure before performing the analyses, and the appropriate caution has been incorporated into the method.

The test solutions showed no increase in impurities over 24 h.

3.2.5. Analysis of available products

The results of the determination of impurities by NMR are presented in Table 10. Individual impurities ranged from trace levels to 0.52%, and total impurities from trace levels to an estimate of 0.82%. Several lots contained small amounts of unknown impurities at trace levels. Three lots, all from manufacturer E contained an unknown impurity which was not present in the other raw materials. This unknown has a triplet at 4.13 ppm, a quartet at 3.72 ppm and a triplet at 0.85 ppm (Fig. 6). From a comparison of the size of these peaks to those of the known impurities in the samples, it is estimated that it is present at a level $> 0.2\%$.

3.3. Validation of HPLC identification of impurities by LC-MS

Sample EE2 was subjected to LC-MS analysis (Table 11), and the total ion and UV chromatograms are provided in Fig. 7. The LC-MS was able to confirm the identity of a number of impurities in this sample and to provide structural information for those impurities which eluted at relative retention times of 0.48, 1.48 and 2.23 on this system. The first two probably correspond to the unknowns eluting at relative retention times of 0.44 and 1.44 on the previously described HPLC system. The impurities which eluted at RRTs of 0.28, 0.30 and 2.23 on the LC-MS system are estimated to be at levels below 0.01% (based on peak height relative to the impurity at RRT 0.51 (compound IV) and were not detected in the original HPLC/UV analysis of this sample. The peak at 11.65 min. (Fig. 7), identified as com-

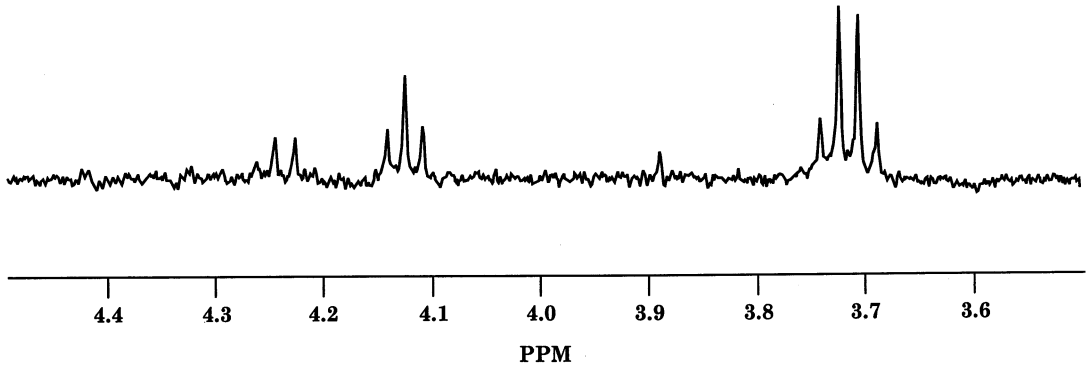

Fig. 6. Portion of the NMR spectrum of fenofibrate raw material EE2 showing the chemical shifts of an unknown impurity in the vicinity of those for impurity X at 4.24 ppm.

pound XII by HPLC/UV, was clearly shown to contain two components (compound XII and a methylated fenofibrate) by LC-MS.

An attempt was made to identify the unknown in samples EE1, EE2 and EE3 which appeared by NMR to be present at levels above 0.2% in these samples. Although these samples also contained an unknown impurity as detected by HPLC at RRT = 0.44 (Table 8), the levels found did not correlate with the levels of the unknown observed by NMR. The peak at 1.1 min may correspond to the unknown compound found by NMR as it had a very low UV response and eluted in the solvent front. The mass spectrum of this peak's substance is presented in Fig. 8. Because this peak eluted at the solvent front there is a strong possibility that it contained more than one component. For example, the m/z 145/147 isotope pattern (if it was one compound) was not repeated for 104/106. Therefore, the peak could have been two components differing by one double bond equivalent.

4. Conclusions

The HPLC and NMR results for related substances in the raw materials analyzed were consistent. The main differences stem from the higher limits of quantitation for the NMR method for several impurities using the conditions described in the method. By increasing the number of scans acquired or by using a higher field instrument, greater sensitivity could be obtained with the NMR method. In addition, the NMR method

Table 11
LC-MS analysis of sample EE2

Retention time (min)	RRT	MH+	Comment
1.10	0.14	145	Large TIC peak very small UV peak
2.20	0.28	?	Drug related: base peak 233 a.m.u.
2.32	0.30	?	Drug related: base peak 233 a.m.u.
2.69	0.35	319	Compound VI
3.70	0.48	405	Possibly fenofibrate–H+COOH on side chain (+44 a.m.u.)
3.92	0.51	247	Compound IV
6.16	0.80	347	Compound X
7.69	1.00	361	Fenofibrate
11.42	1.48	375	Methylated fenofibrate (methyl group on one of the phenyl rings): base peak = 247 a.m.u.
11.66	1.51	447	Compound XII
17.16	2.23	491	Possibly compound XII–H+COOH on side chain (+44 a.m.u.)

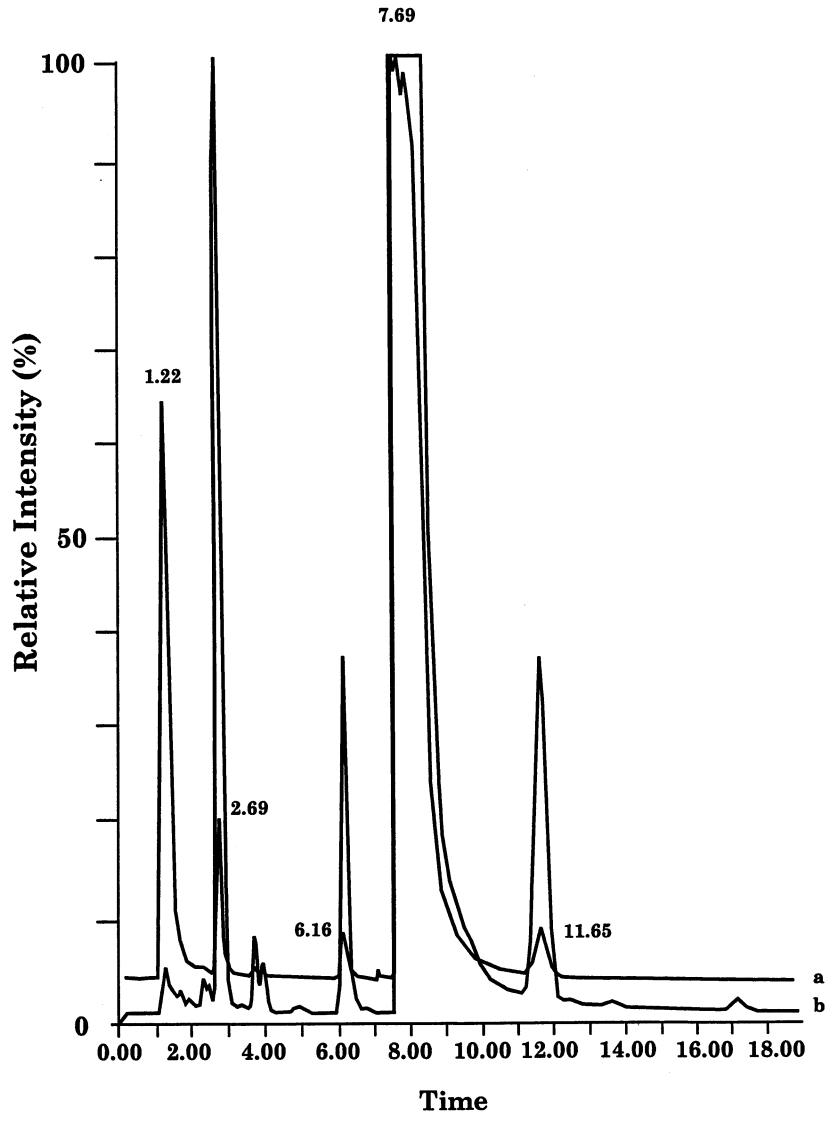

Fig. 7. LC-MS chromatogram of sample EE2 (20 μl injection of a 5.26 mg ml^{-1} solution). Trace a: total ion chromatogram, and trace b: UV detection at 280 nm.

was able to detect some impurities which were not detectable by HPLC: trace levels of compound VII in samples CA1 and ED2 and an unknown impurity in samples EE1, EE2 and EE3. Other advantages of NMR over HPLC include the reduced use of solvents and that signals from unknown impurities provide information which is potentially useful for characterization or identification. The main drawback of this technique is the high capital cost of the instrumentation.

Sample EE2 was not homogeneous in the content of impurity VI, a synthetic intermediate and possible degradation product although the content of other impurities was uniform. The sample was analyzed by both methods in triplicate on three separate days (Table 5). A consistent level of impurity VI could be obtained if the

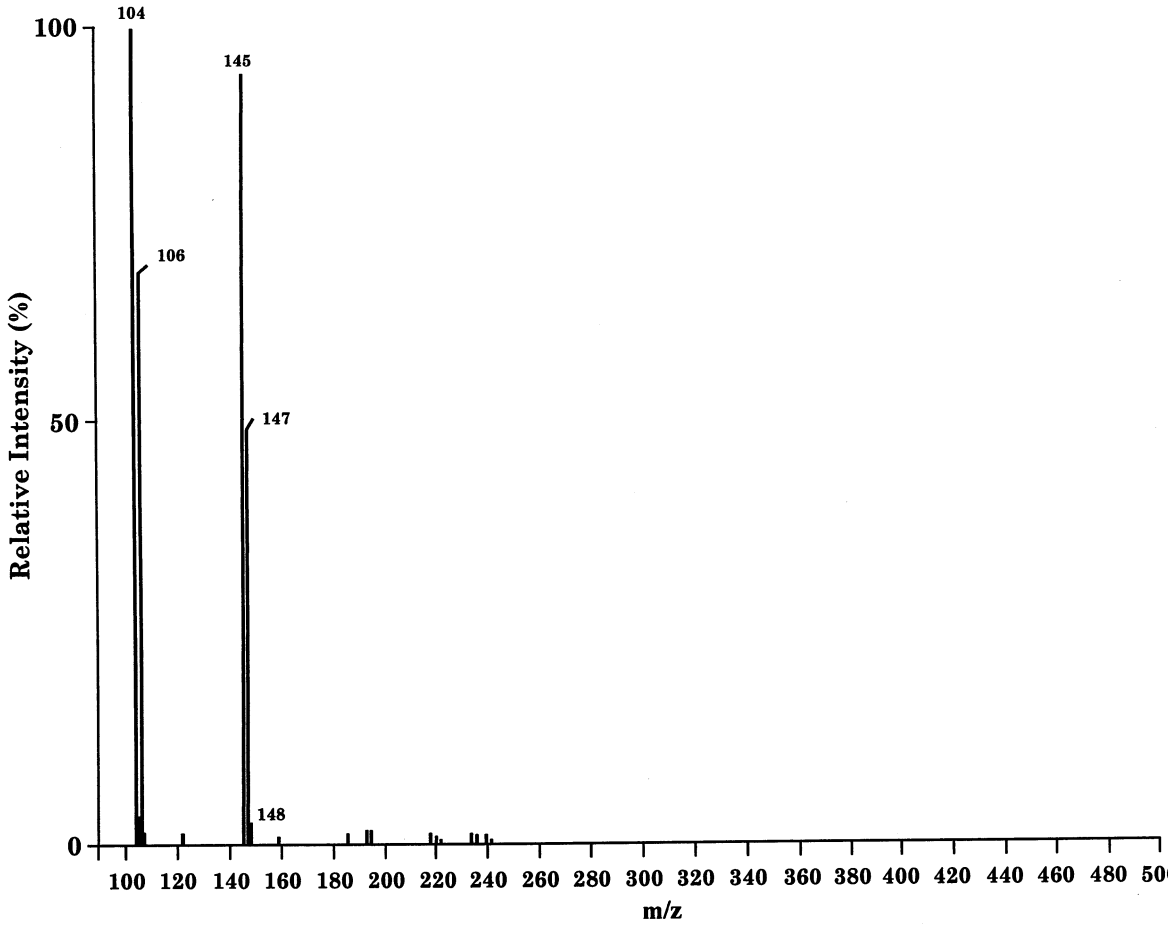

Fig. 8. Mass spectrum of the unknown in raw material EE2 at a retention time of 1.1 min by LC-MS.

sample was ground and mixed prior to analysis (0.24%, triplicate analyses by NMR). We have reported on the non-homogeneity of other drug raw materials in the past [22,23]. Such findings underscore the need for suitable sampling of raw materials and an adequate procedures for investigating these occurrences to determine the cause and extent of the problem and how it relates to the quality of the pharmaceuticals.

The HPLC method has a few advantages over the proposed EP method: it was validated for a larger number of related compounds, resolves compounds which are unresolved by the proposed EP method, and, other than the drug, requires only one reference standard compound rather than three. In addition, this compound is commercially available.

With only slight modifications to the HPLC method, an LC-MS procedure was developed which could be used to confirm the identity of related compounds in samples of raw material and provide structural information on some of the unknowns detected by the HPLC method. The LC-MS procedure was considerably more sensitive than either of the other two methods. Minor components were easily detected, and full scan MS spectra were obtained at concentrations well below 0.01% relative to the drug. The peak identities were confirmed on-line from the full scan mass spectra, and co-eluting peaks (RRT 1.48 and 1.51 by LC-MS or 1.47 by HPLC) were discerned. The major disadvantage is the greater degree of difficulty associated with quantitation which

requires either an isotopically labelled or closely related internal standard.

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