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Development and validation of a single robust HPLC method for the characterization of a pharmaceutical starting material and impurities from three suppliers using three separate synthetic routes

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Abstract

Novel approaches to the development of analytical procedures for monitoring incoming starting material in support of chemical/pharmaceutical processes are described. High technology solutions were utilized for timely process development and preparation of high quality clinical supplies. A single robust HPLC method was developed and characterized for the analysis of the key starting material from three suppliers. Each supplier used a different process for the preparation of this material and, therefore, each suppliers' material exhibited a unique impurity profile. The HPLC method utilized standard techniques acceptable for release testing in a QC/manufacturing environment. An automated experimental design protocol was used to characterize the robustness of the HPLC method. The method was evaluated for linearity, limit of quantitation, solution stability, and precision of replicate injections. An LC-MS method that emulated the release HPLC method was developed and the identities of impurities were mapped between the two methods.

Keywords: Impurity profiles; Impurity map; LC-MS; Automated robustness

1. Introduction

Compound **1** is a key registered starting material for the manufacturing of Orbofiban, a potential antithrombotic drug candidate that attained phase III clinical status. ICH guidelines indicate that impurities at or above 0.1% in the drug substance require identification [1]. The use of high quality starting material was important for the control of impurity levels, the avoidance of new impurities, and the production high purity drug substance.

The analytical method used to assay compound **1** needed to be simple and straightforward. Moreover, the method needed to utilize standard equipment available at the manufacturing site.

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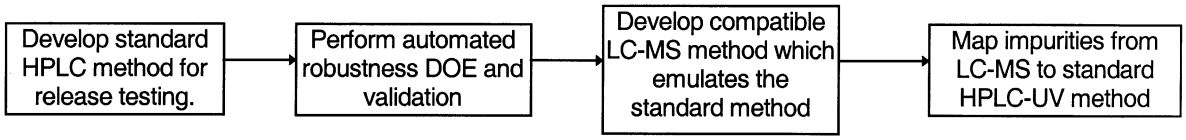


Fig. 1. This is a depiction of the overview analytical strategy used.

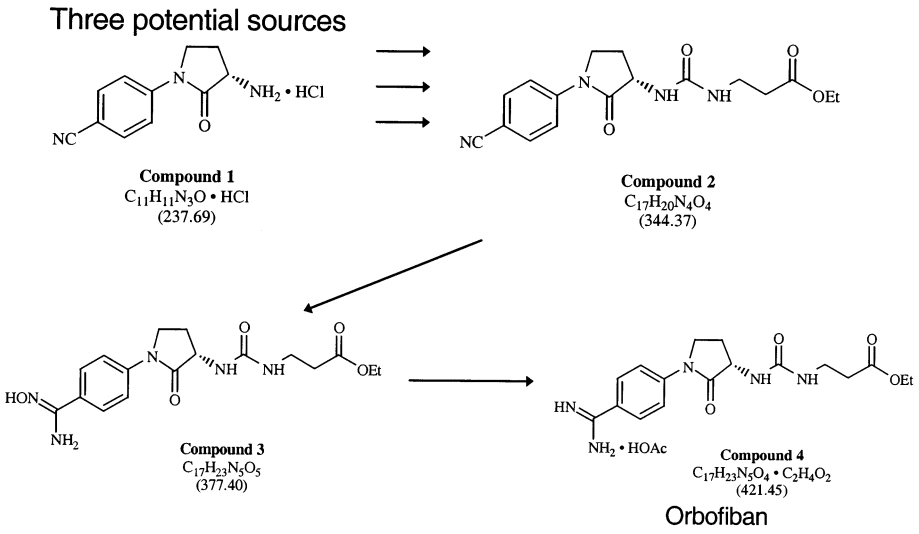


Fig. 2. The synthesis of Orbofiban. Compound **1** was purchased from three suppliers exhibiting unique impurity profiles.

Three vendor sources were identified as suppliers of the material. The goals of this study were to develop a single method for the quantitation of compound **1** and its impurities ($>0.1\%$) from each source, identify the impurities, and to study the fates of impurities in subsequent reactions. Achievement of these goals would permit good process control during manufacture.

Historically, these goals would have been accomplished by tentative identification and preparation or isolation of individual impurities followed by classical techniques of method characterization and validation. The approach detailed here provides an expeditious alternate means of accomplishing these goals by utilizing LC-MS technology. Though numerous examples of LC-MS used for the identification of impurities in pharmaceuticals exist [2–5], this paper links the LC-MS method directly to a non-volatile LC-UV QC method. The overall work process is illustrated in Fig. 1.

Orbofiban was prepared according to the Fig. 2. During the preparation, impurities and potential carryover starting materials were identified from various steps in the process. The structures of these compounds are illustrated in Fig. 3.

2. Experimental, results and discussion

2.1. Equipment and reagents

All solvents used in the HPLC method were of analytical grade. Compound **1** samples were obtained from three vendor sources. Hewlett Packard 1100 HPLC systems were used for this study. The detectors for these systems were UV-diode array or UV-diode array/HP G1946A mass spec. For the mass spec, APCI and ESI were evaluated. A ChemStation was used for instrument control and automated data collection. Linking several methods in sequence was critical for generating

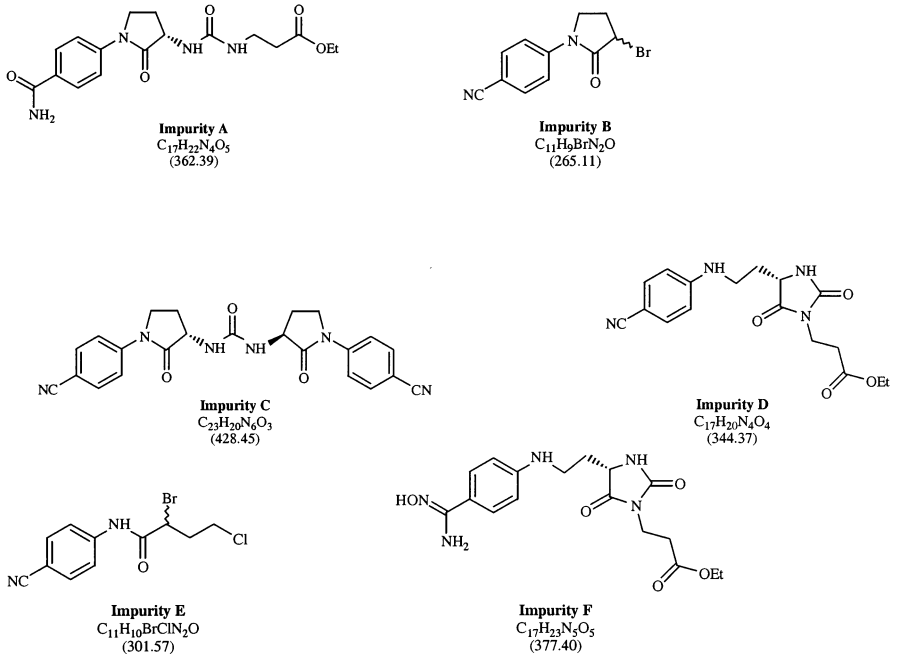


Fig. 3. Potential impurities in Orbofiban drug substance.

results for several experiments rapidly and with minimal user intervention.

2.2. Method development

Compound **1** was studied using reversed-phase chromatography. The initial method development began with a method based on the standard in-house LC-MS method. Two batches from each supplier were studied. Because no markers of impurities were available, six isocratic conditions each monitoring four wavelengths were used to ensure that all impurities at or greater than 0.1% were being detected. Initially, gradient conditions were not used so that artifact peaks would not mask or be mistaken for impurities. Though gradient artifacts are usually distinguishable from impurities via a blank injection, small impurities coeluting with larger artifacts might be overlooked. The isocratic approach avoids these problems. The study was automated in an overnight run by linking the six methods in a sequence. The mobile phase compositions were studied for each of the six samples according to Table 1. A Zorbax

Table 1
Isocratic mobile phase conditions studied using a Zorbax SB-C18, (150 × 3) mm, 3.5 μm column[a]

Condition	% Acetate buffer, pH 4.8	% Methanol
1	10	90
2	30	70
3	50	50
4	70	30
5	80	20
6	90	10

[a] Six methods were created and linked in sequence for rapid method development.

Table 2
Second column study[a]

Condition	% Acetate Buffer, pH 4.8	% Methanol
1	80	20
2	50	50

[a] Isocratic mobile phase conditions studied using an Altima C18 column, 5 μm (250 × 3.2) mm.

Table 3
Column three evaluation[a]

Condition	% Acetate buffer, pH 4.8	% Methanol
1	50	50
2	70	30
3	80	20
4	90	10

[a] Isocratic mobile phase conditions studied using a YMC basic column, C8, 5 μm (3 × 150) mm.

SB-C18, (150 × 3) mm, 3.5 μm column was used with a flow rate of 1.0 ml/min.

The results from conditions 1, 2, and 3 (Table 1) indicated that there were no significant late eluting peaks. The parent peak eluted near or at the void volume with these conditions.

The peak shape was inherently poor so the initial column was replaced by an Altima C18 column, 5 μm (250 × 3.2) mm. The flow rate was 1.0 ml/min. From the first study it was clear that the highest levels of impurities were in vendor A, batch 1 and vendor B, batch 1. Further studies

Table 4
Final HPLC conditions used for the analysis of Orbofiban related compounds

Column	YMC basic, (4.6 × 150) mm, 5 μm		
Wavelength	280 nm		
Buffer	10 mM Phosphate, pH 7.0		
Organic modifiers	Methanol and acetonitrile		
Elution type	Gradient		
Flow rate	1.0 ml/min		
Run time	20 min		
Reequilibration Time	10 min		
Time (min)	% Buffer	% Acetonitrile	% Methanol
Gradient table			
0	80	0	20
8	75	5	20
20	10	20	70

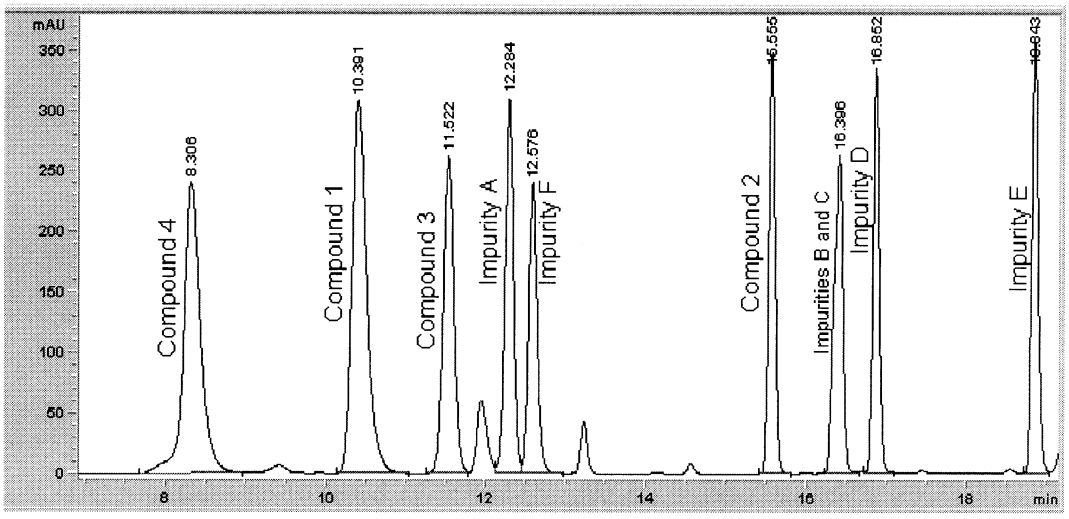


Fig. 4. Chromatogram depicting the resolution of Orbofiban related compounds. The HPLC conditions can be found in Table 4.

Table 5
Final HPLC conditions used for the analysis of compound **1**

Column	Waters Symmetry Shield, (4.6 × 100) mm, 3 μm	
Wavelength	280 nm	
Buffer	10 mM phosphate, pH 7.0	
Organic modifier	Methanol	
Elution type	Gradient	
Flow rate	1.0 ml/min	
Run time	20 min	
Reequilibration time	10 min	
Time (min)	% Buffer	% Methanol
Gradient table		
0	95	5
20	25	75

were limited to these batches. Both samples were studied with two isocratic conditions at four wavelengths according to Table 2, however the peak shape remained poor.

The column was again changed to a YMC Basic, C8 column 5 μm, (3 × 150) mm. Four isocratic conditions were evaluated according to Table 3, however the peak shape was still unsatisfactory. The standard flow rate of 1.0 ml/min was used.

It was now clear that the conditions under study would not be suitable for the original intent. A phosphate buffer pH 7.0 was used to rectify peak shape problems. The organic modifier was also changed to acetonitrile.

Using the same rationale, several isocratic conditions were studied. The parent appeared stable in 85/15 buffer/acetonitrile diluent. When this was completed, work commenced on a gradient system that would account for all major impurity peaks greater than 0.1% that were seen isocratically.

Concurrently, a universal system that would resolve all steps and critical impurities from each step was explored for the following compounds: compounds **1**, **2**, **3** and **4** and impurities **A**, **B**, **C**, **D**, **E**, and **F**. After several attempts, a system (Table 4) was discovered that would resolve all compounds except impurities **B** and **C** (Fig. 4). This was not an issue because it would be impossible for these compounds to exist in the same step. The method was also found to resolve some unknown process impurities.

From this study it was found that a both acetonitrile and methanol were needed to achieve the overall separation. The system was a somewhat complex tertiary gradient system and attempts made to simplify the conditions were

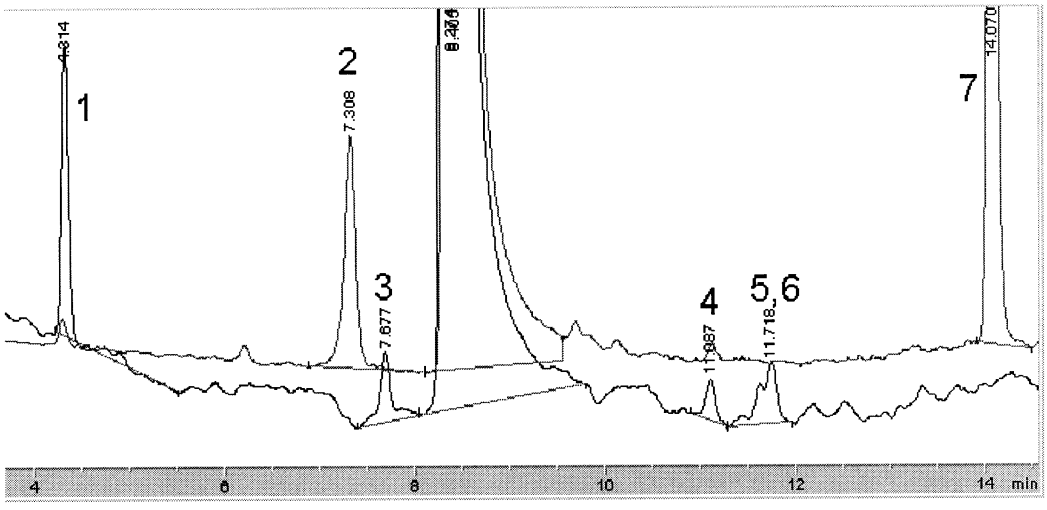


Fig. 5. Example chromatograms of compound **1** from vendors A and B. The phosphate buffer system conditions from Table 4 were used. Impurities 1–7 were identified as significant from the two batches.

Table 6
Robustness D.O.E., $2^{(4-1)}$ plus star point design

Experiment	Vendor order	pH	Flow rate (ml/min)	Temperature (°C)	% Methanol (starting and ending points)
1	A, B	6.8	0.9	25	6–90
2	B, A	6.8	1.1	25	4–60
3	B, A	7.0	1.0	20	5–75
4	A, B	7.2	1.1	25	6–90
5	B, A	6.8	0.9	15	4–60
6	A, B	6.8	1.1	15	6–90
7	A, B	7.0	1.0	20	5–75
8	B, A	7.2	1.1	15	4–60
9	A, B	7.2	0.9	15	6–90
10	B, A	7.2	0.9	20	4–60
11	B, A	6.8	1.0	20	5–75
12	B, A	7.2	1.0	20	5–75
13	B, A	7.0	0.9	20	5–75
14	B, A	7.0	1.1	20	5–75
15	B, A	7.0	1.0	15	5–75
16	A, B	7.0	1.0	25	5–75
17	B, A	7.0	1.0	20	4–60
18	A, B	7.0	1.0	20	6–90

unsuccessful. Using the above system, two batches from each vendor were re-examined to establish impurity profiles.

A decision was made to abandon this route, and to instead, focus on previous simpler gradients in which co-elution of some compounds across steps but not within steps takes place [6]. This method employed the Waters Symmetry Shield column, (4.6 × 100) mm, 3 μm, that appeared to have better efficiency than that of the YMC Basic column. See Table 5 and Fig. 5.

2.3. Diluent studies

At this point it was noticed that the choice of diluents had a large effect on the area of an early eluting impurity in Vendor B samples. A 10 μl injection volume was studied. In general, the greater the difference between the diluent and mobile phase the worse the peak shape of the early impurity. Though a lower injection volume would improve this, worse overall method precision would need to be considered. For the time being, continuing studies utilized a 75/25 buffer/methanol mobile phase diluent. Two samples of each batch were examined and the area % was tabulated.

2.4. Experimental design of robustness

The method was at a point suitable for robustness studies to be conducted. For the design of experiment (D.O.E.), pH, composition, flow rate of the mobile phase as well as column temperature were used as variables. A $2^{(4-1)}$ plus star points was used as the design [7]. The two lowest purity samples from vendors A and B were used to evaluate the method. No significant impurities were detected in samples from vendor C. The effect of HPLC conditions on number of peaks detected, resolution, plates, and tailing were studied. Plates and tailing were only studied for one batch since these values were independent of batch. Seventeen distinct methods were created and linked in random order on the HP1100 ChemStation for an automated experiment. The center point was studied in duplicate resulting in a total of 18 experiments (Table 6). The results from the experiments were tabulated in Table 7a and b.

Irrespective of the deviations of the conditions, the method exhibited good robustness as seen in the results. There was little change in vendor B sample resolution, area %, tailing, and plates and vendor A area %. The resolution for Vendor A

sample fluctuated, but was always well over the acceptable limit. The number of peaks detected was variable for both batches and was attributed, in general, to low level peaks at the limit of detection ($\sim 0.02\%$). The number of peaks $> 0.1\%$ was nearly constant, the exceptions were due to a peak with an area % very close to 0.1% from vendor A.

The results were supplied to our Statistical Department for analysis [7]. It was confirmed that most of the factors did not influence the analytical results. The gradient variation had the most influence, however it was found that changing the gradient improved some results but at the cost of others. Typically this manifested in improved resolution/fewer peaks detected or decreased resolu-

Table 7
Results of robustness experiments for vendors A and B.

Experiment	Resolution of compound **1** to closest impurity	Compound **1** Area %	Number of peaks detected and (number of peaks > 0.1%)	Tailing factor	Plates
(a) Results for vendor A					
1	5.86	97.08	10 (5)		
2	12.01	97.12	9 (4)		
3	8.29	97.10	8 (4)		
4	7.18	97.05	10 (4)		
5	10.31	97.21	8 (4)		
6	7.36	97.04	9 (5)		
7	8.13	97.05	9 (4)		
8	11.87	97.18	6 (4)		
9	5.39	96.99	9 (4)		
10	10.07	97.11	7 (4)		
11	8.72	97.14	8 (4)		
12	8.44	97.02	9 (4)		
13	7.44	97.02	8 (4)		
14	9.45	97.08	8 (4)		
15	8.67	97.08	8 (4)		
16	8.35	97.05	8 (4)		
17	11.14	97.05	8 (4)		
18	6.64	97.00	8 (5)		
(b) Results for vendor B					
1	5.80	98.71	5 (3)	1.132	36014
2	6.06	98.73	4 (3)	1.135	30496
3	5.80	98.73	5 (3)	1.127	33785
4	5.55	98.64	7 (3)	1.117	34391
5	6.01	98.78	4 (3)	1.176	31784
6	5.53	98.64	7 (3)	1.131	27734
7	5.71	98.67	7 (3)	1.125	34658
8	5.44	98.78	3 (3)	1.115	33154
9	5.22	98.62	7 (3)	1.124	40436
10	6.07	98.69	6 (3)	1.125	41438
11	5.87	98.67	6 (3)	1.138	31733
12	5.63	98.68	6 (3)	1.127	36970
13	5.74	98.67	6 (3)	1.145	36973
14	5.63	98.67	6 (3)	1.130	30750
15	5.60	98.69	5 (3)	1.145	32290
16	5.90	98.64	7 (3)	1.118	35709
17	5.91	98.69	5 (3)	1.140	33265
18	5.44	98.62	7 (3)	1.124	34458

Table 8
Precision of results for various sample preparations of compound **1**

Target preparation	Replicate	Response factor
20 mg–100 ml	1	0.546
20 mg–100 ml	2	0.554
20 mg–100 ml	3	0.544
20 mg–100 ml	4	0.542
20 mg–100 ml	5	0.549
	% RSD (1–5)	1.0%
10 mg–50 ml	1	0.542
10 mg–50 ml	2	0.540
5 mg–25 ml	1	0.540
5 mg–25 ml	2	0.545
	Total % RSD (all samples)	0.82%

Table 9
Single point biases (residuals) from the linearity plot

Level (%)	% Bias-D, system 1	% Bias-D, system 2
0.05	−15.67	17.44
0.05	−3.78	14.38
0.1	−5.17	1.08
0.1	−10.56	−3.48
0.2	−5.02	−2.94
0.2	−4.21	−1.79
0.5	−4.40	−2.74
0.5	−3.54	−3.20
1.0	−2.75	−0.19
1.0	−2.96	−1.87
80	0.15	0.95
80	0.32	1.51
90	0.37	1.02
90	0.33	0.44
100[a]	−0.11	−0.14
100[a]	0.11	0.14
110	0.04	0.58
110	0.04	−0.76
120	0.14	−0.80
120	−0.34	−1.27

[a] Single point standard.

tion/more peaks detected. This indicates that the nominal condition for the method were near optimal and robust.

2.5. Further sample preparation studies

At this point, the diluent was further studied. One 0.1% impurity in the vendor B batches was found to be unstable in the 75/25 buffer/methanol diluent. Three additional diluents were studied: 85/15 buffer/acetonitrile, 50/50 water/acetonitrile, and 50/50 water/methanol. Both buffer-based diluents were found to degrade the impurity peak of interest. The water/acetonitrile diluent resulted in distorted peak shape of an early impurity. A marker of the amide of compound **1** [8], now available, confirmed the peak shape observations and identity of this impurity peak.

The 50/50 water/methanol diluent looked to be the most promising until the sample from vendor A sample was studied. Here, detection of early impurities was lost due to baseline disturbances. One final diluent, a 75/25 water/methanol was also studied. Overall, this was the most promising diluent and the method evaluation would continue with this.

Precision was evaluated using five replicates of a 20 mg–100 ml preparation. In addition to five samples prepared using the above scenario, the study was extended to include two preparations at 10 mg–50 ml and 5 mg–25 ml. Acceptable precision was obtained for all preparations (Table 8).

2.6. Method validation

The linearity of the method was studied with a target concentration of 0.3 mg/ml compound **1**. For the assay level, 80, 90, 100, 110 and 120% standards were prepared. For the impurity level linearity, 0.05, 0.1, 0.2, 0.5 and 1.0% of target were studied. The samples were injected on both a low (system 2) and high (system 1) dead volume HPLC systems. Five injections of the 0.05% standard were injected to establish LOQ. The % RSD was 5.2 and 6.3% for systems 1 and 2, respectively. Because reference standards of the impurities were not available, accuracy was determined using the biases for the main peak (Table 9). For example the % Bias-D of the 1.0% standard, system 1, run 1, was −2.75%. This indicates that the response of the 1.0% standard was slightly lower (97.25%) than the theoretical response

(based on the 100% standard). From the biases and correlation coefficient it was determined that no significant deviations from linearity were observed on either HPLC system. The correlation coefficients for full fit were 1.0000 and 0.9999 for systems 1 and 2, respectively. The results suggest that the 100% standard could be used for quantitation at low levels with acceptable accuracy.

2.7. LC-MS impurity map

Work began for the development of an LC-MS compatible method. Elimination of the buffer resulted in poor peak shape and detectability problems for the impurities. The phosphate buffer was then replaced by an acetate buffer at the same pH. After slight modifications of the gradient conditions, the impurity profiles were similar irrespective of the employment of acetate or phosphate buffers (Figs. 5 and 7). The efficiency was somewhat better with the phosphate system which would be the preferred system for the QC function. The overall strategy is depicted in Fig. 6.

To further substantiate that elution order of impurities was preserved between methods, UV spectra of the larger impurities from both conditions were compared. The UV comparison is illustrated in Fig. 8. Due to low levels, UV spectra were not obtained for impurities 3, 5, and 6.

Procedures were developed using both APCI and electrospray sources. The MS detector was operated in the positive mode with a typical scanning range of 100–1000 m/z. Mass spectra were obtained from all of the significant impurities greater than 0.1%. These were compared with the structures that had previously been deduced by

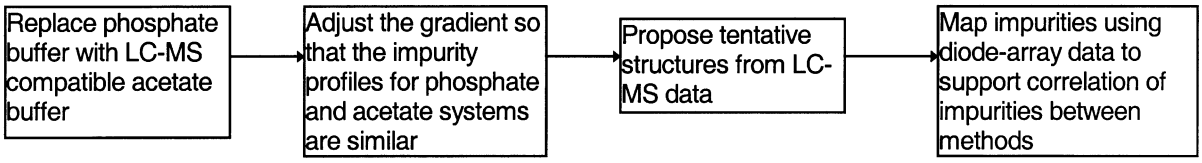


Fig. 6. This illustrates the work process used to identify and map impurities for LC-MS acetate system and LC-UV phosphate system. This schematic provides additional detail to steps 3 and 4 of Fig. 1.

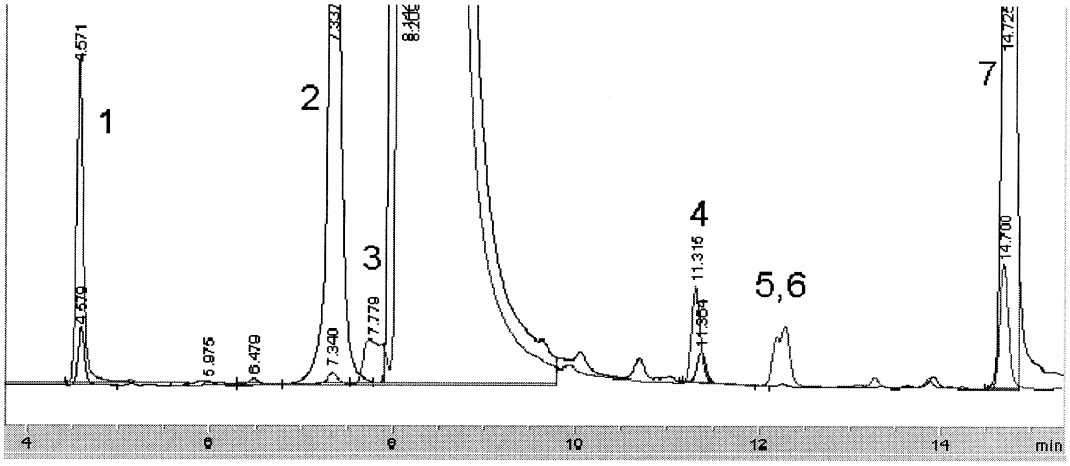


Fig. 7. Example chromatograms of compound **1** batches from vendors A and B. This figure displays an overview of significant impurities from both sources. The acetate buffer in place of the phosphate buffer and modified gradient conditions were used. This mobile phase is suitable for LC-MS with a comparable impurity profile to the LC-UV method. The HPLC conditions are listed in Table 11.

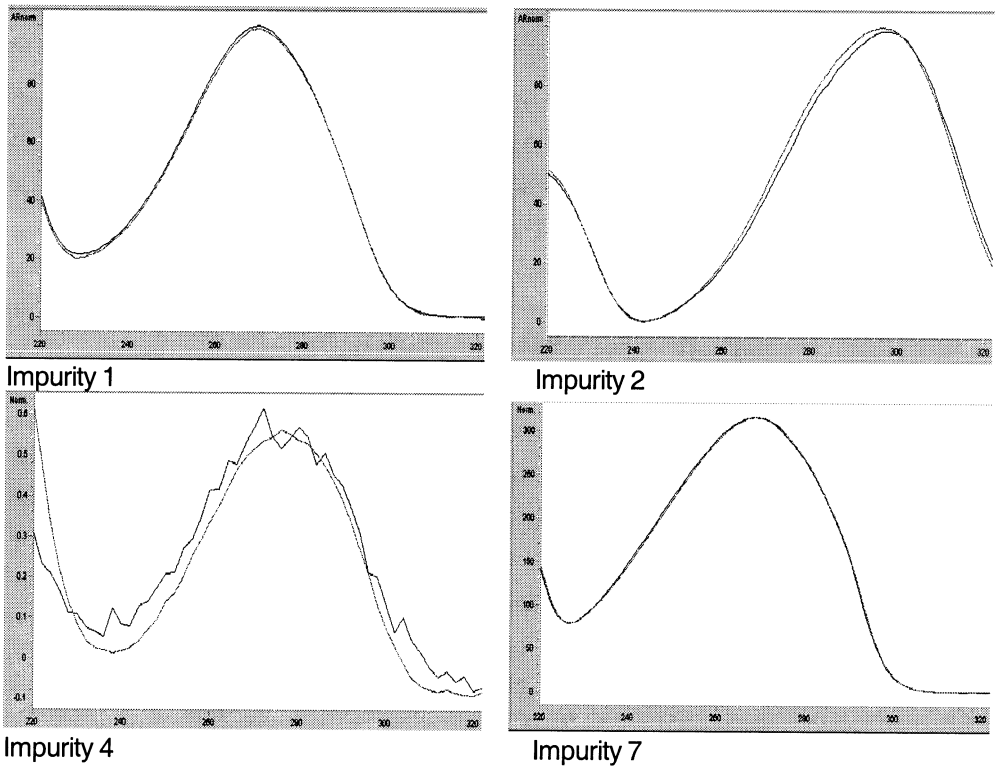


Fig. 8. Comparison of UV spectra from the phosphate and acetate HPLC methods to ensure peak tracking between systems. This data further substantiates that the elution order of impurities has not changed.

the Mass Spectrometry Group [9]. From this exercise it was clear that some impurities that had been identified were actually at a very low level, and impurities 2 and 3 (Table 10) had not been identified. By obtaining mass spectra of these compounds, the structure of the larger of these was now tentatively deduced [9]. Due to interferences, it was suggested that the other impurity be run on a triple quadrupole instrument. Correlation of all the major impurities back to the phosphate system without using markers or reference standards was accomplished. The largest instance (area %) of each impurity in representative samples was tabulated in Table 10. The final conditions for the LC-MS method are detailed in Table 11.

3. Conclusions

A robust HPLC method to evaluate compound **1** raw material was developed and validated. Experimental design techniques were successfully employed as part of the method characterization. The power of the ChemStation was utilized to generate 18 experiments, completely automated and within a two day time frame.

LC-MS instrumentation was used to identify and map impurities back to the routine HPLC method without the use of expensive reference standards. Useful information was generated using a fast, scientifically sound approach which allowed the overall objectives of characterizing the starting material to be carried out.

Table 10
Map of impurities from LC-MS to LC-UV methods

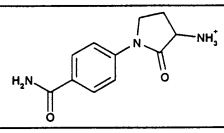
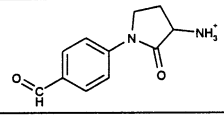
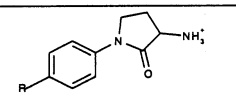
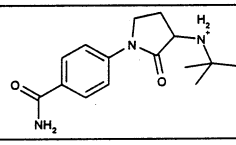
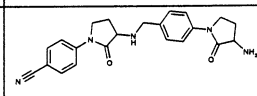
Impurity #	Retention Time (Min)	Mass	Proposed Structure [9]	Maximum Area % Observed
1	4.3	219		0.28
2	7.4	204		0.26
3	7.7	247	Unknown	0.10
4	11.0	234	 where R = 59 or a combination adding up to 59 anywhere on the ring	0.19
5	11.8	276		0.08
6	11.8	372	Unknown	0.08
7	14.1	389		0.98

Table 11
The LC-MS mobile phase conditions are stipulated below

Column	Waters Symmetry Shield, (4.6 × 100) mm, 3 μm	
Wavelength	280 nm	
Buffer	10 mM Acetate, pH 7.0	
Organic modifier	Methanol	
Elution type	Gradient	
Diluent	75/25 water/methanol	
Flow rate	1.0 ml/min	
Run time	20 min	
Reequilibration time	10 min	
Time (min)	% Buffer	% Methanol
Gradient table		
0	100	0
20	30	70

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