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Comparison of supercritical fluid chromatography and reverse phase liquid chromatography for the impurity profiling of the antiretroviral drugs lamivudine/BMS-986001/efavirenz in a combination tablet

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ABSTRACT

Dual and triple combinations of antiretroviral drugs are a cornerstone of human immunodeficiency virus type 1 (HIV-1) treatment. Supercritical fluid chromatography (SFC) and reverse phase liquid chromatography (RPLC) methods have been developed for the impurity profiling of a prototype combination tablet containing three such drugs: lamivudine, BMS-986001 and efavirenz. Separation by SFC was achieved using a Princeton 2-ethyl pyridine stationary phase and a mobile phase B consisting of methanol with 10 mM ammonium acetate and 0.1% isopropyl amine. This combination of mobile phase additives was required for both the separation of minor components and to minimize peak tailing of the active pharmaceutical ingredients (APIs). Separation by RPLC was achieved using a Discovery HSF5 stationary phase and a mobile phase consisting of 10 mM ammonium acetate, pH 5.5 and methanol. Mobile phase gradient elution was employed in each case to elute components with a wide range of polarities. Both these methods were found to have advantages and disadvantages. Out of the three APIs and 13 possible impurity/degradation products selected, all were resolved by RPLC. By SFC, 15 peaks were resolved with one co-eluting pair and a high degree of orthogonality was achieved relative to RPLC. A more even distribution of peaks across the separation space, a non-sloping baseline and fewer system peaks were significant advantages associated with the SFC method. Particular attention had to be paid to optimizing the reverse phase diluent strength/initial mobile phase composition to avoid distortion of the peak shapes for early eluting components. This was not an issue with SFC, as the diluent of choice (methanol) was also the solvent of choice (in combination with ≤20% water) for the dissolution of the triple combination tablet. As with RPLC, SFC was found to exhibit the required sensitivity for successful quantitation of potential impurities/degradation products at the 0.05–0.1 area% level.

1. Introduction

The inherent sensitivity, high selectivity and reproducibility of modern RPLC with ultraviolet (UV) detection have made this the technique of choice for the impurity analysis of active pharmaceutical ingredients (APIs) which often contain components that are both hydrophilic and hydrophobic in nature. However, despite the extensive variety of RP stationary phases and the range of optimization parameters available, such as control of pH, temperature and mobile phase polarity, it may not be possible to completely separate all components in a complex mixture using this technique. At times, a compromise separation is achieved. This being a balance between achieving sufficient retention (and selectivity) for very polar (hydrophilic) species, which are often degradation products, while at the same time having reasonable retention times for the less polar (more hydrophobic) components of the mixture. The analysis of mixtures of antiretroviral drugs, including nucleoside reverse transcriptase inhibitors (NRTIs), and their potential degradation products, particularly in the development of combination dosage forms, is particularly challenging in this respect. Dual and triple combinations of antiretroviral drugs, including zidovudine, efavirenz, abacavir, lamivudine, emtricitabine and tenofovir, are a cornerstone of human immunodeficiency virus type 1 (HIV-1) treatment [1]. Despite the relative success of antiretroviral therapy, emergence of drug resistant HIV-1 variants continues to be the major cause for treatment failure. BMS-986001 (previously known as OBP-601, or Festinavir) is currently under development by Bristol-Myers Squibb Co. It is a novel nucleoside analog with potent anti-HIV-1 activity and decreased cytotoxicity [2]. The structure is shown in Fig. 1.

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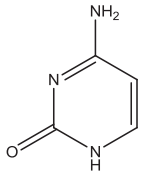
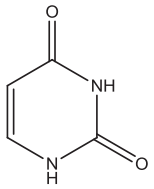
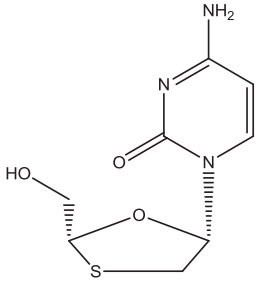
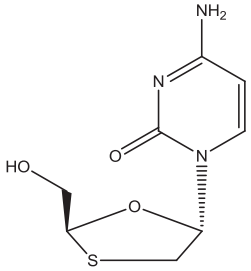
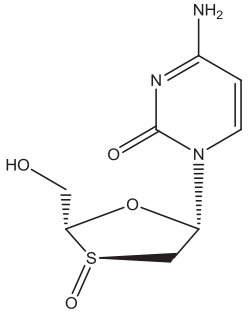
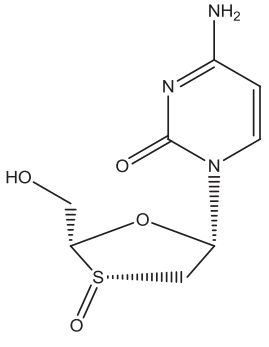
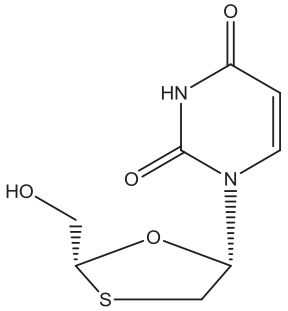
Table 1
Lamivudine drug substance and drug product related substances.

Designation	Structure	Formation
Cytosine (Imp-I)		Acid, base stress
Uracil (Imp-II)		Acid, base stress
Lamivudine (Lam)		N/A
Lam-D (Diastereomer)		Heat
Lam-III		Peroxide stress
Lam-IV		Peroxide stress
Lam-V		Acid, base stress

Data compiled from Refs. [13,15,16,18].

The majority of literature reports on the analysis of either dual or triple combinations of anti-HIV agents (as either drug substances, or as drug product formulations), have focused on the assay of these drugs by RPLC with UV detection [3–8]. This involves relatively straight forward RPLC method development, in that it only requires that the mixture of APIs be reproducibly separated from each other. However, the development of a RPLC–UV method capable of separating all potential degradation products, in addition to the APIs, a so called "stability indicating method", is much more challenging, particularly for triple combination dosage forms. Development of this type of method for combinations of antiretroviral agents have been reported for lamivudine and stavudine (efavirenz) [9], lamivudine, zidovudine, and nevirapine [10], lamivudine, zidovudine and abacavir [11] and lamivudine, zidovudine and TMC278.HCl (rilpivirine) [12]. Monographs describing LC–UV methods are also included in the United States Pharmacopeia for lamivudine [13] and efavirenz [14] drug substances and for lamivudine and stavudine tablets [15]. In addition, the 4th edition of the International Pharmacopeia describes LC–UV methods for lamivudine [16] and efavirenz drug substances [17].

The forced decomposition behavior of lamivudine has been studied [18]. In total, five major degradation products were formed, which could be separated by RPLC on a C_{18} column using gradient elution. These products were also identified by LC–MS/TOF and MS^n and their designations, structures and formation conditions are listed in Table 1. A complete list of known and potential impurities for lamivudine is included in the 4th edition of the International Pharmacopeia [16].

Several stability indicating RPLC methods have been developed and validated for efavirenz and its related substances in both drug substance and in a capsule formulation [19–21]. The kinetics and mechanism of hydrolysis of efavirenz has also been studied in aqueous solution [22]. The designations, structures and degradation pathways are summarized in Table 2.

A complementary approach to the analysis of triple combination NRTIs is to employ the alternative selectivity provided by SFC,

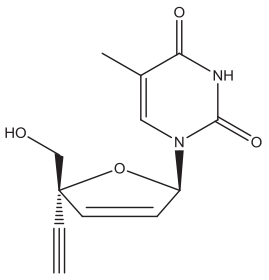


Fig. 1. Chemical structure of BMS-986001(also known as OBP-601, or Festinavir).

Table 2
Efavirenz drug substance and drug product related substances.

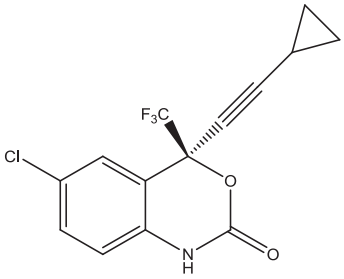
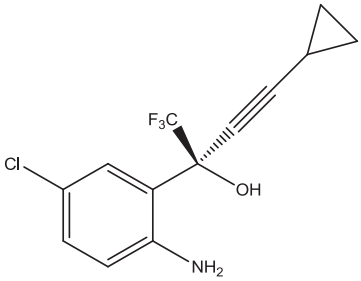
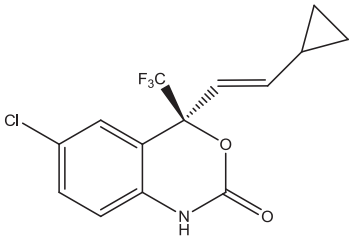
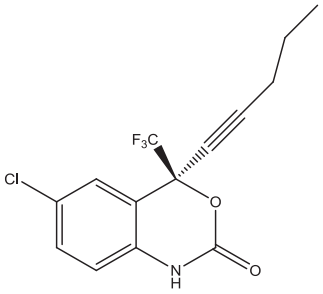
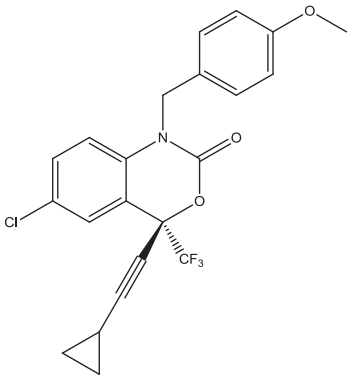
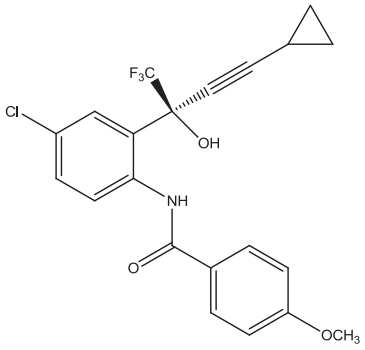
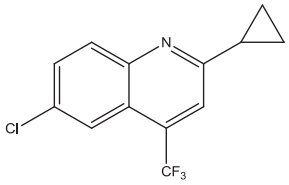
Designation	Structure	Formation
Efavirenz		N/A
SD573[a]		Base stress
SR695		Potential in-process impurity
SP234		Potential in-process impurity
SE563		Potential in-process impurity

Table 2 (*Continued*)

Designation	Structure	Formation
SW965		Potential in-process impurity
SM097		Base and photolytic stress

Data compiled from Refs. [14,17,19].
[a] Note, SD573 is the primary degradation product of efavirenz.

which is a form of normal phase (NP) chromatography. In SFC, the same mobile phase can be used with both polar and non-polar stationary phases, which provides analytical capabilities not readily accessible with LC [23]. That is, as expressed by Taylor [24], NP and RP modes are limiting behaviors, and they are bridged in SFC. However, in the past SFC has not been used extensively in the regulated pharmaceutical industry for the impurity profiling of APIs due to the relatively poor sensitivity and reproducibility of the technique compared to RPLC [25]. However, with new advances in instrumentation [26,27], it has been recently demonstrated that good quality impurity profile data, that is, data yielding single digit % RSDs for impurities present at less than 0.1 area%, can be obtained by analytical SFC [28]. Furthermore, for the pharmaceutical compounds studied, the elution profiles were found to be generally orthogonal in nature to that obtained with RPLC [28].

In this study we report a comparison of SFC and RPLC for the impurity profiling of the antiretroviral drugs lamivudine, BMS-986001 and efavirenz in a prototype tablet formulation. This report will focus particularly on the method optimization hurdles that had to be overcome in each case and will critically compare the final separation efficiencies achieved with each technique. Interestingly, to our knowledge, no literature results have been published on the use of SFC with UV detection for the separation of either single, dual, or triple combinations of anti-HIV drugs.

2. Experimental

2.1. Instrumentation

An Agilent Technologies (Waldbronn, Germany) 1200 LC was converted to operate as a super critical fluid chromatograph by addition of a Fusion A5 Evolution conversion module (Aurora SFC Systems, Redwood City, CA, USA) as documented elsewhere [26,27]. The LC consisted of a G1312B binary SL pump, a G1367C Hip autosampler, a G1316B column compartment, a G1379B degasser and a G1315C SL diode array detector. The injection valve was fitted with a 5 μL external loop and the diode array detector was fitted with an Aurora 13 μL 10 mm path length 400 bar flow cell (P/N D004). The other instrumental details, such as the

plumbing arrangements, the column switching valves employed, and the pump compressibility settings are the same as previously described [28]. Also, as previously described [28], the post column eluent (for all columns employed) was directed through the left-hand-side heat exchanger (3 μL internal volume) of the column compartment to pre-condition the fluid temperature prior to delivery to the detector. This temperature was maintained at 43 °C, which was found to be the optimum temperature for minimal noise (≤0.05 mAu peak-to-peak) at a flow rate of 2–2.5 mL/min with 20% methanol and a BPR setting of 160 Bar. Once established, this temperature was fixed and not used further as a tuning parameter. The instrument was further modified as described by Alexander [29] to allow blends of methanol with up to three additional solvents and/or additives to be delivered to pump B on-the-fly. Such an on-line arrangement allows for greater flexibility in method development, that is, in the "fine-tuning" of the method once the stationary phase and gradient conditions have been established. RPLC was performed with a Waters Acquity H-class UPLC system (Milford, MA, USA), which was equipped with a quaternary solvent manager, a sample manager with flow-through needle design, a column manager and tunable UV detector.

2.2. Chemicals

Instrument grade carbon dioxide (99.99%) was purchased from Airgas East (Piscataway, NJ) in 200 size cylinders. Analytical HPLC grade methanol and acetonitrile were purchased from EMD Chemicals (Gibbstown, NJ, USA) and water was purified in-house using a Milli-Q UV Plus purification system (Millipore Corp., Billerica, MA, USA). Isopropyl amine (≥99.5%) and ammonium acetate (≥99.5%) were obtained from Sigma–Aldrich, (Saint Louis, MO, USA) Lamivudine (Lam) was obtained from Lonzeal Pharmaceuticals Co., Ltd. (Shijiazhuang City, China). Lamivudine diastereomer (Lam-D) was obtained from the USP (USP Reference Standard B, Rockville, Maryland, USA). Bristol-Myers Squibb BMS-986001 [2], efavirenz (EFV), efavirenz related substances, SD573, SR695, SP234, SW965, SE563, SM097 were obtained in-house. Prototype dual (Lam/BMS-986001) and triple (Lam/BMS-986001/EFV) combination tablets were also manufactured in-house. Thymine, cytosine and uracil were obtained from Sigma–Aldrich (Saint Louis, MO, USA). Samples of lamivudine API were stressed with base to yield markers for Lam-V and with peroxide to yield markers for Lam-III and Lam-IV as described by Bedse et al. [18]. Thymine is the primary degradation product of BMS-986001.

2.3. Sample preparation

A 16 component Lam/BMS-986001/EFV and related impurities/degradation products marker stock solution was prepared as follows: A lamivudine/BMS-986001 tablet was heat stressed for ~five days at 80 °C under humid conditions. The tablet was then disintegrated in ~20 mL of water by a combination of ultrasonic agitation and shaking. Approximately 600 mg of EFV API and ~80 mL of methanol was added to this solution and the resulting mixture shaken for a further 10 min. The supernatant liquid, containing 3 mg/mL each of BMS-986001 and lamivudine and 6 mg/mL of EFV, was removed by a combination of centrifugation and filtration (0.45 μm PVDF filter). This degradation procedure for the tablet produced the following markers: cytosine, Lam-III, Lam-IV, Lam-V and thymine. To ~4 mL of this solution appropriate amounts of SD573, SR695, SP234, SW965, SE563, SM097, Lam-D and uracil standards were added to give individual levels of ~0.5 area%.

For both SFC and RPLC analyses the marker stock solution was further diluted (10-fold) to give marker solution A which contained final BMS-986001 and lamivudine concentrations of 0.3 mg/mL and an EFV concentration of 0.6 mg/mL. In the case of SFC, the solvent used was methanol, which resulted in a final sample diluent composition of ~2% water in methanol (v/v). For RPLC analysis the solvent employed was a methanol/water mixture (50:50, v/v) which resulted in a final sample diluent composition of ~47% water in methanol (v/v). Both the final 16 component marker solutions (SFC and RPLC) were filtered using a 0.2 μm PVDF filter prior to analysis.

Sample analyses were carried out on a Lam/BMS-986001/EFV triple combination tablet solution, which was prepared as follows. The tablet was ultrasonically agitated in ~20 mL of methanol:water (10:90, v/v) for a period of 20 min, followed by 10 min of mechanic shaking, until it was fully disintegrated. Approximately 140 mL methanol was then added and the volumetric flask mechanically shaken for a further 15 min, after which time the volume was adjusted to 200 mL by the addition of methanol. The resulting solution was filtered sequentially through 0.45 μm and 0.2 μm PVDF filters. The final composition was ~90% methanol:10% water (v/v) and the API concentrations were 1 mg/mL BMS-986001, 1.5 mg/mL Lam and 3 mg/mL EFV. This solution was used as is for the SFC analysis, but was further diluted (5-fold) using a mixture of 40/60 methanol:water (v/v) for RPLC analysis. This reduced the final API concentrations to 0.2 mg/mL BMS-986001, 0.3 mg/mL lamivudine and 0.6 mg/mL EFV in a sample diluent composed of 50/50 methanol:water (v/v).

2.4. Stationary phases

The SFC stationary phases used in this study for screening purposes were the same as previously described [28]. All columns dimensions were 150 mm × 4.6 mm I.D. The particle size was 5 μm, apart from the 2-ethyl pyridine and PPU columns (3 μm) and the HILIC column (2.7 μm Fused Core®). The following RPLC phases were screened for initial column selection: Discovery HSF5 column (Sigma–Aldrich, Saint Louis, MO, USA), Kinetex™ 2.6 μm PFP column (Phenomenex, Torrance, CA, USA), Zorbax SB-Aq column, (Agilent Technologies, Santa Clara CA, USA), Dionex Acclaim Trinity P1 column (Dionex, Sunnyvale, CA, USA), SIELC Primesep 100, SIELC Obelisc R columns (SIELC Technologies, Prospect Heights, IL, USA), Acquity UPLC HSS T3 column, XSelect™ HSS PFP column, Acquity UPLC CSH C18 column, (Waters, Milford, MA, USA).

2.5. Final chromatographic conditions

Optimized conditions for the separation of Marker Solution A:

RPLC method: column: Discovery HS F5, 4.6 mm × 150 mm, 3 μm, mobile phase A: 10 mM ammonium acetate, pH 5.5; mobile phase B: methanol; flow rate: 0.8 mL/min; column temperature: 35 °C; linear gradient: 5% B 0–0.7 min, 5–95% B 0.7–20.5 min, 95% B 20.5–23.8 min, 95–5% B 23.8–25.1 min, 95% B 25.1–30.4 min; UV detection: 260 nm; injection volume: 3.5 μL.

SFC method: column: 2-ethyl pyridine, 4.6 mm × 150 mm, 3 μm, mobile phase B: methanol containing 10 mM ammonium acetate with 0.1% isopropyl amine; flow: 2.5 mL/min; BPR: 160 bar; column temperature: 25 °C; linear gradient: 0% B 0–1 min, 0–12% B 1–20 min; UV detection: 260 nm; injection volume: 5 μL

3. Results and discussion

3.1. RPLC method development

As most practitioners will be familiar with the elements of RP method development, only some of the more significant challenges that had to be overcome to generate a stability indicating RP method for a triple combination NRTI pharmaceutical product will be discussed here. The general strategy employed was the same as that described for the RP separation of complex pharmaceutical samples [30,31], that is, automated column screening,

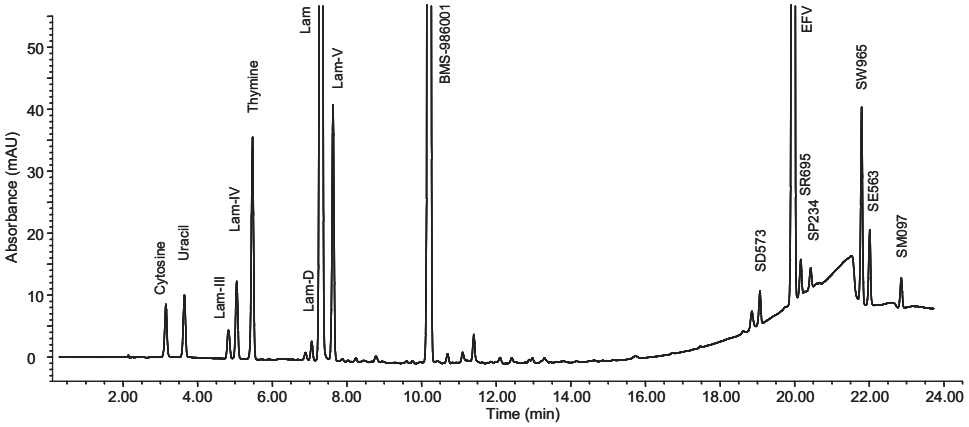


Fig. 2. RPLC separation of components in Lam/BMS-986001/EFV and related impurities/degradation products marker solution (marker solution A). Column: Discovery HS F5, 4.6 mm × 150 mm, 3 μm, mobile phase A: 10 mM ammonium acetate, pH 5.5; mobile phase B: methanol; flow rate: 0.8 mL/min; column temperature: 35 °C; linear gradient: 5% B 0–0.7 min, 5–95% B 0.7–20.5 min, 95% B 20.5–23.8 min, 95–5% B 23.8–25.1 min, 95% B 25.1–30.4 min; UV detection: 260 nm; injection volume: 3.5 μL.

initial optimization of the most critical chromatographic parameters, computer modeling, and experimental verification/tweaking of the predicted separation. In this particular case, the challenges included: initial column selection (to retain cytosine and uracil with acceptable peak profiles for quantitation, while separating the more hydrophobic efavirenz related components in a reasonable amount of time), selection of a suitable diluent and tablet disintegration medium to overcome the limited solubility of efavirenz, elimination of any diluent solvent strength effect for early eluting peaks and finally the optimization of the separation between several critical pairs in what is a complex mixture of potential impurities and degradation products (see Tables 1 and 2). The optimized separation of the 16 components in the Lam/BMS-986001/EFV and related impurities/degradation products marker solution (marker solution A) is shown in Fig. 2. Three critical pairs were found to be very challenging with respect to obtaining baseline separation, these being, lamivudine/Lam-D, lamivudine/Lam-V, and efavirenz/SR695.

3.1.1. Column selection

The RP method development was initiated using only the dual combination of lamivudine/BMS-986001 and related impurities, as the successful retention of cytosine and uracil, both highly polar compounds, was considered to be one of the keys to the column selection process. The literature methods for the separation of lamivudine related compounds typically utilize either C_{18} [12,13,16,18], or C_8 [9] stationary phases. However, these methods, which all employ 4.6 mm × 250 mm × 5 μm columns, have run times of ≥30 min. Whereas, the goal of this study was to generate a method using the more modern 3.0 mm × 150 mm × 3 μm column format and employ a relatively shorter run time. A literature search of column vendor data bases was performed and a number of columns shown to have selectivity for cytosine and uracil were obtained for evaluation. These are listed in Section 2.4. These columns were screened using appropriate generic mobile phases and gradient programs. Only the Discovery HSF5 column was found to provide an acceptable combination of retention, resolution and peak shape for the components present in the dual combination product (data not shown).

3.1.2. Selection of diluent

Due to the presence and nature of the excipients, a highly aqueous (≥80%) dissolution medium is initially required to disintegrate the triple combination tablet (see Section 2.3). However, while efavirenz is soluble in methanol (~700 mg/mL) and acetonitrile (>200 mg/mL), it is practically insoluble in water. Thus, after the initial disintegration of the tablet, the organic content of the extraction medium has to be increased to ≥50% by addition of either methanol, or acetonitrile. With regard to the second dilution step, which is required to prepare the injected sample, at least 50% methanol was found to be required to achieve a concentration of 0.6 mg/mL (this being the target concentration after dissolution of 600 mg of efavirenz in a combination tablet). However, the mobile phase starting conditions for this separation are highly aqueous (95% mobile phase A), as is typical for most RPLC impurity profiling separations. Based on the above discussion/conditions, several constraints were placed on the selection of a diluent for the RPLC separation: the limited solubility of efavirenz in water, the influence of solvent strength/solvent effect on the early eluting peaks (cytosine and uracil), and the solvent combination required to provide an effective disintegration medium for the tablet. A compromise final sample diluent composition that satisfied these constraints was determined to be methanol: water (50:50, v/v). Also, no improvement in the peak shapes of the early eluting components were obtained as acetonitrile was progressively substituted for methanol in the diluent (data not shown).

3.1.3. Influence of mobile phase pH

During the initial column screening studies, the use of low mobile phase pH had been ruled out due to problems encountered with co-elution and peak broadening. Based on the pKa of cytosine (4.2), mobile phase pH values ranging from 5.0 to 6.8 were examined for the pH optimization studies. The results showed (data not shown) that the mobile phase pH has a significant effect on the separation between lamivudine and Lam-D, and between lamivudine and Lam-V, but only a minor effect on the separation between Lam-III and Lam-IV (see Table 1 for the structures of these compounds). Furthermore, pH values at the lower end of this range were found to favor the separation between lamivudine and Lam-D, but had a negative impact on the separation between lamivudine and Lam-V. Thus, a pH value of 5.5 was selected as a compromise. Note that samples of Lam-III, Lam-IV and Lam-V were generated in-house by degrading lamivudine API and then adding these components to the retention time marker mixture (see Section 2.5 for experimental conditions). The mobile phase pH (in the range studied) was found to have no effect on the separation of efavirenz and its related impurities.

3.1.4. Optimization of gradient program

In order to separate the wide polarity range of components in the 16 component marker sample a linear gradient of 5–95%B over a minimum gradient time (t_g) of 20 min was required (see Fig. 2). However, the eluted components were not well dispersed

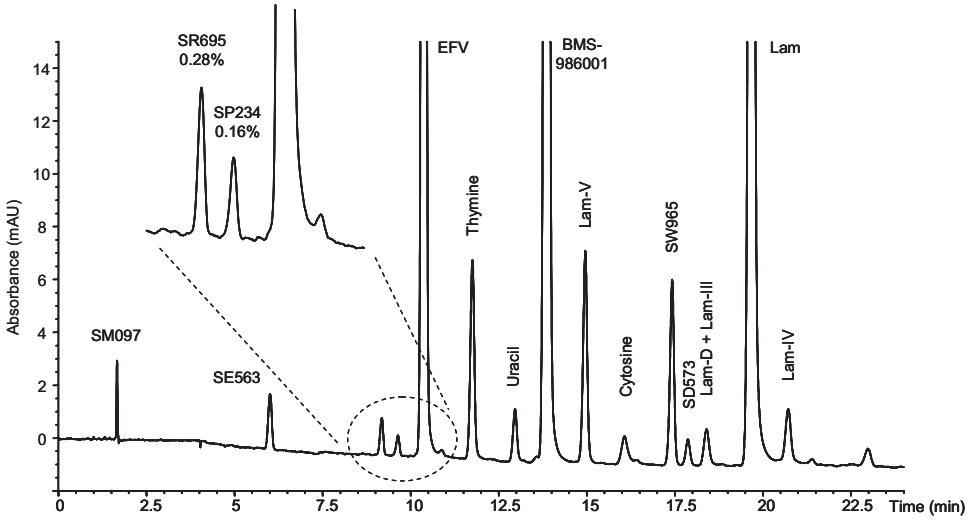


Fig. 3. Optimized SFC separation of components in marker solution A. Column: 2-ethyl pyridine, 4.6 mm × 150 mm, 3 μm, mobile phase B: methanol containing 10 mM ammonium acetate plus 0.1% isopropyl amine, flow: 2.5 mL/min, BPR: 160 bar, column temperature: 25 °C, gradient: 0%B–1 min hold, linear to 12% B–20 min. UV detection at 260 nm, injection volume: 5 μL.

across the separation space (see later discussion with respect to comparison with SFC results). That is, all lamivudine/BMS-986001 components eluted closely together in a relatively narrow retention time window of 3–10.5 min, whereas efavirenz and its related impurities/degradants all eluted in a significantly later window of 18.5–23.5 min. Secondly, although all components are eluted in a reasonable time, some of them are only just baseline resolved. In order to see if the separation between these components could be further optimized, the use of a segmented gradient was investigated. That is, two separate gradient optimizations were performed using DryLab® [32], one using a mixture of lamivudine/BMS-986001 and their related impurities and the other using efavirenz and its related impurities and the results combined to give a segmented gradient. However, this gradient program did not significantly improve upon the separation achieved with a simple linear gradient (data not shown).

3.1.5. Influence of column temperature

The influence of column temperature on the RPLC separation of the marker solution A was examined in detail (data not shown). Over the range from 25 to 45 °C the retention time of all components was found to progressively decrease with increasing temperature. That is, no switches in peak elution order with temperature were observed. A higher temperature (45 °C) was found to improve the separation between the critical pair lamivudine/Lam-V and was also found to improve the resolution between Lam-IV and thymine. A higher temperature was also found to improve the separation between the critical pairs efavirenz/SR695 and SW965/SE563. Conversely, a lower temperature (25 °C) was found to favor the separation between the critical pairs Lam-III/Lam-IV and Lam-D/lamivudine and was also found to improve the separation between SP234 and SR695. Thus a temperature of 35 °C was employed as a compromise.

3.2. SFC method development

As was the case with RPLC, the SFC method development was initiated using only the dual combination of lamivudine/BMS-986001 and related impurities to simplify the tracking of peaks. These column screening efforts were undertaken using generic conditions as previously described [28], except in this instance 10 mM ammonium acetate was added to mobile phase B (methanol) as this concentration of buffer salt has been shown to be effective at suppressing peak tailing [33]. The most promising separation was obtained at a flow rate of 2.5 mL/min with the 2-ethyl pyridine phase and a gradient of 5–20% B in 20 min (data not shown). These conditions were then as the starting point for optimization of the conditions to separate the components in marker solution A. After gradient and temperature optimization the separation shown in Fig. 3 was obtained. All components, except Lam-D and Lam-III, were effectively separated and a more even distribution across the separation space obtained compared to RPLC (see plot of retention factor (k') versus retention time in Fig. 4). The sample diluent composition for marker solution A consisted of ~2% water (v/v) in methanol (see Section 2.3). This level of water was not found to have any significant impact on the peak shapes of early eluting components. The addition of 0.1% isopropyl amine (in addition to 10 mM ammonium acetate) was required to minimize peak tailing, particularly for cytosine and lamivudine. However, unlike the peak shapes obtained with the RPLC separation, residual tailing could not be completely eliminated, particularly for lamivudine.

Further method development was focused primarily on trying to obtain separation of the Lam-D/Lam-III critical pair. With respect to the influence of pressure, no significant improvement in the separation efficiency, or the signal-to-noise (S/N) ratio, was noted over the BPR range 160–200 bar. At BPR pressures <160 bar, the

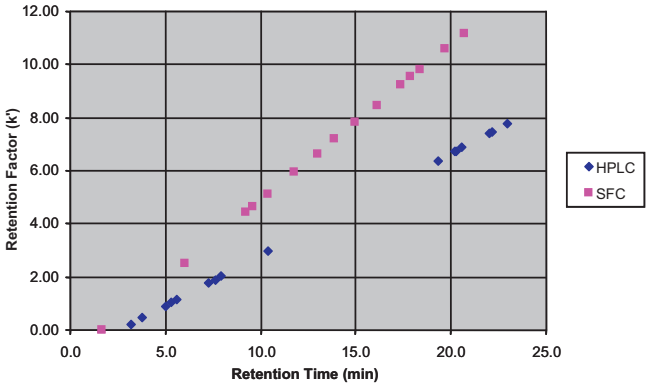


Fig. 4. Plots of retention factor (k') versus retention time for the optimized HPLC and SFC separations shown in Figs. 2 and 3 respectively. Note that the SFC separation more effectively disperses the components across the available separation space.

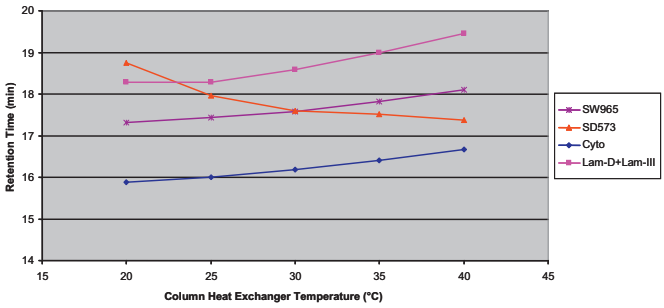


Fig. 5. Influence of temperature on the SFC separation of components Lam-D/Lam-III, Cytosine, SD573 and SW965. See Fig. 3 for other chromatographic conditions.

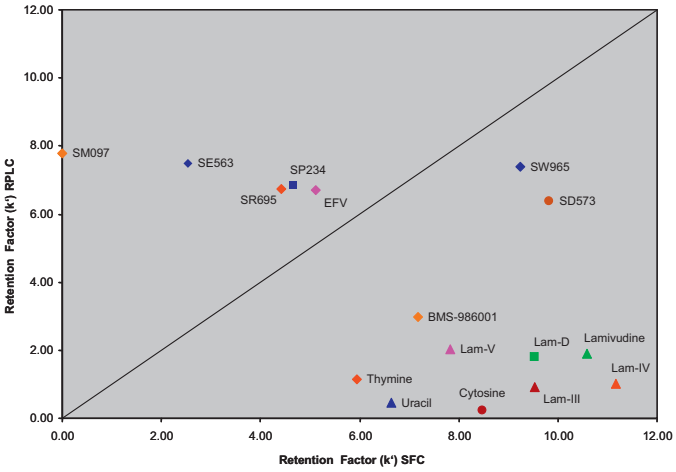


Fig. 6. Plot of HPLC versus SFC retention factors (k') for the optimized separations shown in Figs. 2 and 3 respectively. The condition where the two retention factors are equal (i.e. no orthogonality) is denoted by the inclined 45° line. Note the high level of orthogonality of the SFC separation.

resolution between the closely eluting components SW965, SD573 and Lam-D/Lam-III was found to progressively reduce with decreasing pressure, whereas the separation of all other components was unaltered. Ashraf-Khorassani and Taylor [34] have reported on the influence of water on the SFC separation of four nucleoside bases using a 2-EP stationary phase. They found that incorporation of a fixed amount of water (up to 5%) into the alcohol modifier yielded a striking improvement in peak shape and intensity for adenine and cytosine, whereas, thymine and uracil were found to yield relatively sharp peaks with, or without water as an additive. They also found that either the addition of 5% water to 5 mM AA produced an improvement in the peak shape for adenine and cytosine that was greater than just the impact of these additives used singly. In a previous study, [28] we have shown than that the addition of just 0.1% of water to mobile phase B can have a significant influence on selectivity when impurity profiling pharmaceutical compounds by SFC. Unfortunately, in this study, the addition of up to 1% water in combination with 10 mM ammonium acetate, was found to decrease the separation of the two peaks corresponding to the components Lam-D/Lam-III and SD573, although the peak tailing for cytosine was further reduced (data not shown). The influence on selectivity of up to 10% of acetonitrile in mobile phase B (MeOH/10 mM ammonium acetate) was also examined, but was found to have no impact on the co-elution of Lam-D and Lam-III (data not shown).

3.2.1. *Influence of column temperature*

The influence of SFC column temperature on the separation of selected components of marker solution A is shown graphically in Fig. 5. Over the range from 20 to 40 °C the retention time of all components was found to progressively increase with increasing temperature, except for compound SD573, which was found to behave conversely. At 20 °C, SD573 elutes just after Lam-D/Lam-III, whereas as the temperature is progressively increased to 40 °C the retention time progressively decreases such that SD573 now elutes before SW965. This behavior was not observed under RPLC conditions, where the retention times of all analytes decreased with increasing temperature. As explained by West et al., temperature can impact retention behavior in SFC conditions in one of two basic ways [35]. Classical SFC retention behavior is for retention to increase with an increase in temperature, and this is usually attributed to the lower fluid density (and hence lower elution strength) of the mobile phase as the temperature is increased. However, when the back-pressure is maintained at 150 bar, or more, the fluid density increases with increasing co-solvent content and the fluid is closer in density to that of a liquid (0.7–0.8 g/cm^3). Under these conditions, if the temperature is increased, then the solute solubility in the mobile phase also increases and hence the retention time decreases. This is also typical HPLC behavior. Interestingly, even though we are operating at the higher backpressure condition, our results, except for compound SD573, are consistent with classical SFC retention behavior.

3.2.2. *Orthogonality of separation: SFC versus HPLC*

Relative to RPLC, a high degree of orthogonality was achieved for this sample set with SFC (see plot of k'_{SFC} versus k'_{RPLC} in Fig. 6). Thus the critical pairs Lam-D/Lam, Lam-III/Lam-IV and efavirenz/SR695, which were difficult to resolve by RPLC, were well resolved by SFC. Furthermore, the expected polar degradation products, cytosine, thymine and uracil, which were difficult to retain with good peak shape by RPLC, were well retained by SFC. However, SFC could not resolve Lam-D from Lam-III and SM097 was poorly retained.

3.2.3. *Analysis of triple combination tablet by RPLC and SFC*

Using the methods previously developed, the impurity profile of a prototype Lam/BMS-986001/EFV triple combination tablet was obtained using each technique. The chromatographic results are shown in Fig. 7 and the area% values of the impurities detected are tabulated in Table 3. Note that the increased peak widths of the APIs in the SFC chromatogram (compared to the RPLC separation) are due to the greater sample loading (7-fold, see later discussion). Although the ICH reporting limit (RL) for impurities is normally ≥0.05 area% [36], in this case, to provide a more extensive comparison of the two techniques, the reporting level (RL) was reduced to ≥0.02 area%. SFC was found to provide more than adequate sensitivity (S/N ratio of 20) for accurate quantitation of peaks at this RL, although the bench-mark RPLC results were superior (due primarily to the lower baseline peak-to-peak noise levels of ≤0.01 mAU for RPLC versus ≤0.03 mAU for SFC). It must be noted that it is not the intent here to provide a strict comparison between the absolute sensitivities of the two techniques. Besides the inherent differences in the design of the respective flow cells and detectors of the two instruments, different sample loadings were employed. A 5-fold dilution of the sample was required prior to RPLC analysis, in order to reduce the diluent strength (see Section 3.1.2), while the SFC sample was undiluted (see Section 2.3). Slightly different injection volumes were also employed, that is, 5 μL for SFC and 3.5 μL for RPLC. However, to put the results in Table 3 in perspective, the absolute peak area responses of the three API peaks have been included as a footnote for each technique.

On inspection of Fig. 7, it is apparent that both chromatograms exhibit characteristics that complicate the accurate quantitation of impurities at this very low level (0.01–0.05 area%). In the case

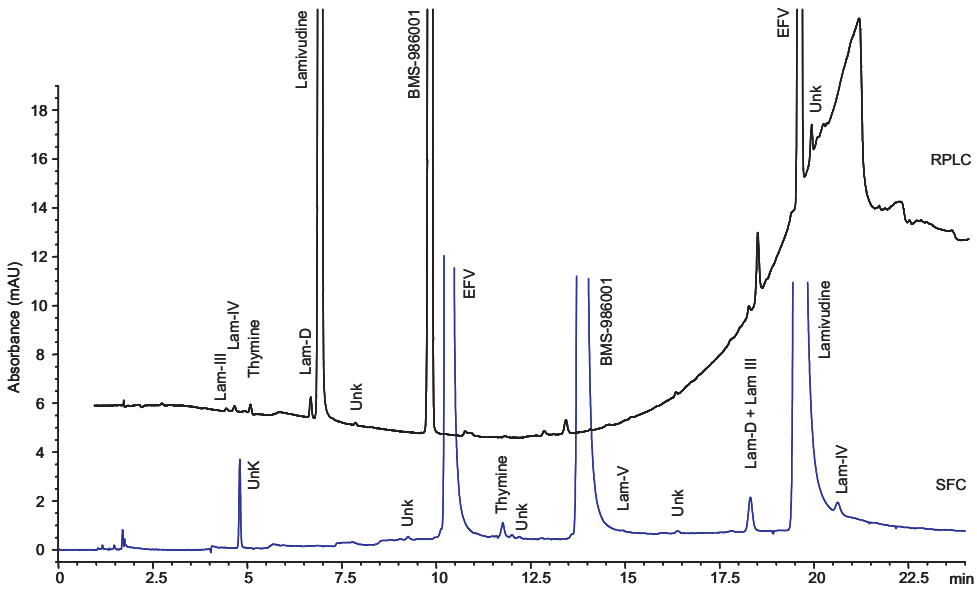


Fig. 7. SFC and RPLC analyses of a prototype Lam/BMS-986001/EFV triple combination tablet. To allow an accurate visual comparison to be made between the two chromatograms they have been displayed on exactly the same absorbance and retention time scales, however, the RPLC chromatogram has been off-set on both axes for clarity. Identified components are labeled and the area% values are given in Table 3. Unk = unknown component. All other unlabelled peaks, in both the RPLC and SFC chromatograms, were present in the respective blanks.

Table 3
Peak area% and signal-to-noise (S/N) ratios for selected impurities detected in the analyses of a prototype Lam/BMS-986001/EFV triple combination tablet by SFC and RPLC. The peak area% difference was calculated from area% $_{SFC}$ − area% $_{RPLC}$. The S/N ratios reported were calculated using peak-to-peak baseline noise values obtained at the same RT for a blank injection. n/r = not relevant, n/a = not applicable.

Component ID	RPLC peak area (%)	SFC peak area (%)	Peak area diff. (%)	S/N ratio SFC	S/N ratio RPLC
Lamivudine	38.07	37.7	0.4		
Efavirenz	33.44	33.74	0.3		nr
BMS-986001	28.33	28.40	0.1		
Lam-D	0.05	n/a	n/a	n/a	78
Lam-III	0.01[a]	n/a	n/a	n/a	9
Lam-D+Lam-III	n/a	0.07	0.01	47	n/a
Thymine	0.02	0.02	0	20	42
Total Unk. Imp. above RL	0.08	0.07	0.01	n/a	n/a

[a] This value has been included, even though it is below the RL selected (≥0.02 area%), so that a comparison can be made to the SFC result. Absolute peak area responses (in mAU × s) for Lam, BMS-986001 and EFV were 2378, 1768 and 2088 respectively for RPLC and 5252, 4435 and 5874 respectively for SFC.

of RPLC, the baseline rise due to the gradient program, required to elute the significantly more hydrophobic EFV related components, is severe. There is also a significant baseline disturbance at ~21.5 min, as well as a number of relatively intense system peaks. Although the SFC chromatogram has a non-sloping baseline and less system peaks, the inherent peak-to-peak baseline noise is higher and the peak tailing is more severe due to the higher sample loading. This is particularly evident in the case of lamivudine and impacts the quantitation of Lam-IV. Despite these drawbacks the peak areas for both the known and total unknown impurities were in good agreement between the two techniques (see Table 3).

4. Conclusions

Traditionally, only RPLC methods have been employed to perform the impurity analysis for either single, dual, or triple combinations of antiretroviral drugs. In this study, we have compared the development of both RPLC and SFC methods to impurity profile a prototype combination tablet containing three antiretroviral drugs: lamivudine, BMS-986001 and efavirenz. Both methods developed for this purpose were found to have advantages and disadvantages. The degree of separation achieved was slightly superior by RPLC, even though the separation of several critical pairs had to be addressed and the method development/optimization was more time consuming. Whereas, the alternative selectivity, a more even distribution of peaks across the separation space, a non-sloping baseline and less system peaks were significant advantages associated with the SFC method. Although only one critical pair was present in the SFC separation (Lam-III/Lam-D), it was unfortunate that this was not found to be resolvable by either manipulation of temperature, pressure, mobile phase composition, gradient time, or use of additives. Clearly, in this instance, a more selective SFC stationary phase would have been beneficial. This reinforces our earlier observation [28] that it is challenging to find SFC conditions that can separate *all* of the components in the impurity profiling of complex pharmaceutical products.

In the case of the RPLC separation, particular attention had to be paid to optimizing the diluent strength/initial mobile phase composition to avoid distortion of early eluting peaks such as cytosine and uracil, while at the same time maintaining the separation of the significantly more hydrophobic efavirenz related components. These challenges were not an issue with the SFC separation as, firstly, cytosine and uracil were generally well retained with all the phases screened, and secondly, the diluent of choice (methanol) was also the solvent of choice (in combination with <20% water) for the dissolution of the triple combination tablet. This also allowed the drug loading to be easily increased for SFC

to counteract the inherently higher peak-to-peak noise present in the chromatogram. Peak tailing, however, was a significant method development challenge for SFC that was not an issue with the RP separation. This was reduced by use of a combination of additives in mobile phase B (10 mM ammonium acetate and 0.1% isopropyl amine), however, it was particularly apparent that the peak widths and tailing factors for the three APIs are still greater than was the case for the RPLC separation due to the higher sample loading. Finally, as with RPLC, the new generation of SFC instrumentation was found to exhibit the required sensitivity for successful quantitation of potential impurities/degradation products at the 0.05–0.1 area% level.

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