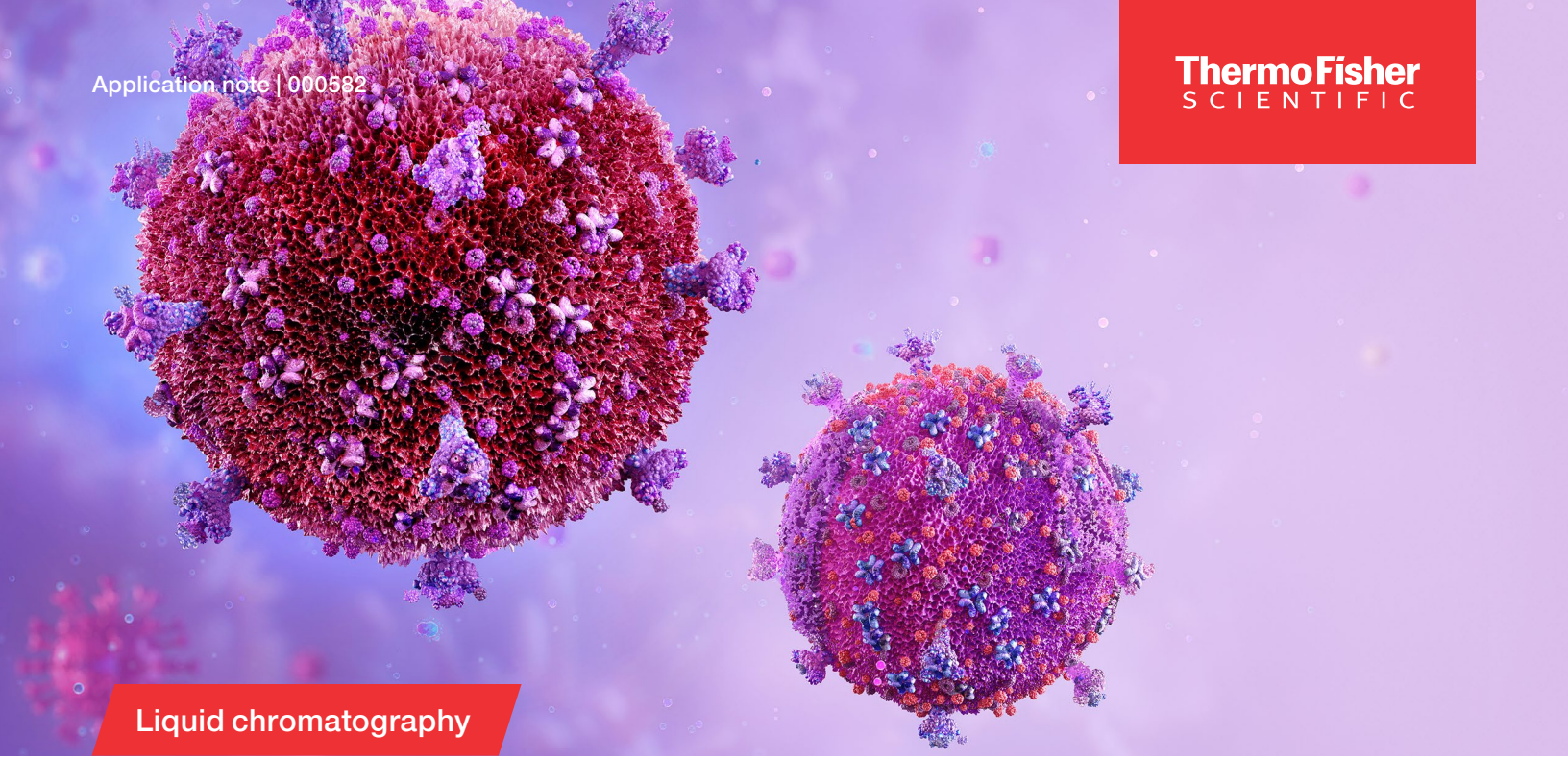

Application note | 000582

Liquid chromatography

Development and validation of a HPLC-DAD method for simultaneous determination of abacavir, lamivudine, dolutegravir, and their related compounds in Triumeq

Authors

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Keywords

HPLC, method development, method validation, abacavir, lamivudine, dolutegravir, anti-HIV drug, Triumeq

Application benefits

- A systematic reversed-phase HPLC method development approach for improved productivity
- Simple, accurate, and reproducible quantitative method for the anti-HIV drug Triumeq™
- Low limits of detection and quantitation for Triumeq-related compounds
- Thermo Scientific™ Vanquish™ Core HPLC and Thermo Scientific™ Acclaim™ 120 C18 column deliver reliable and precise quantitative results

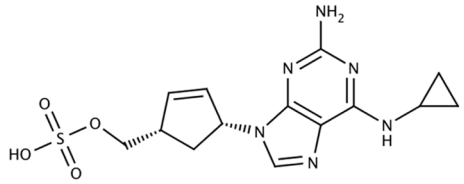
Goal

To develop a sensitive and reproducible HPLC-DAD method for the separation and quantitation of abacavir, lamivudine, dolutegravir, and their 11 related compounds in Triumeq by employing a systematic method development approach.

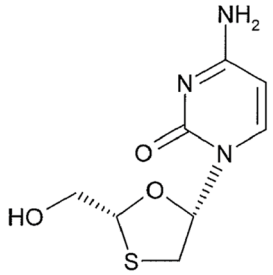
Introduction

Combination drugs, such as several antiretroviral agents in one single drug product, are commonly used for HIV therapy due to their high efficacy and convenience.[1] A “once-daily” tablet for the therapy of HIV-1 with the trade name Triumeq was approved by the US Food and Drug Administration (FDA) in 2014 and is the best-selling and most

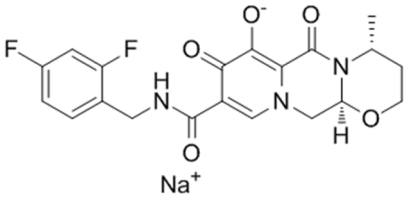
competitive anti-HIV drug on the market.[2,3] This formulation is composed of three active pharmaceutical ingredients (APIs) in each tablet—abacavir sulfate 600 mg, lamivudine 300 mg, and dolutegravir sodium 50 mg (Figure 1). Analysis of this combination drug is challenging due to the presence of more than 20 components, including APIs, impurities, and excipients. Without a systematic guideline, developing an effective and robust HPLC method for such a complicated mixture is difficult and time-consuming.



Abacavir sulfate



Lamivudine



Dolutegravir sodium

Figure 1. Chemical structures of abacavir sulfate, lamivudine, and dolutegravir sodium

In this application note, a stepwise RP-HPLC method development approach is demonstrated. A Thermo Scientific™ Vanquish™ Core HPLC system with a Diode Array Detector (DAD) and a Thermo Scientific™ Acclaim™ 120 C18 (3 µm, 4.6 × 150 mm) column were used. Gradient elution was employed using 20 mM ammonium formate (pH=5) in water and methanol (with 0.01% formic acid). A total of 21 peaks including 3 APIs, 11 impurities, and other unknown compounds were well separated in 37 min. The method was validated and deemed suitable for routine quantitative analysis of Triumeq.

Experimental

Instrumentation

Vanquish Core Quaternary HPLC system consisting of:

- Vanquish Core System Base (P/N VC-S01-A)
- Vanquish Quaternary Pump CN (P/N VC-P21-A)
- Vanquish Split Sampler CT (P/N VC-A12-A)
- Vanquish Column Compartment C (P/N VC-C10-A)
- Vanquish Diode Array Detector CG (P/N VC-D11-A)

Software

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) version 7.3 was used.

Reagents and consumables

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Methanol, HPLC grade (P/N A452-4)
- Fisher Scientific™ Acetonitrile, HPLC grade (P/N A998-4)
- Fisher Scientific™ Formic acid, Optima™ LC/MS grade, HPLC grade (P/N A117-50)
- Ammonium formate, LC-MS grade, ≥99% purity
- Ammonium bicarbonate, ≥99.5% purity
- Abacavir, lamivudine, dolutegravir and their related compounds reference standards (Table 1)
- ViiV Healthcare UK Limited, Triumeq film coated tablets, purchased from Huifan pharmacy, Shanghai, China

Sample preparation

Triumeq drug product solution

Ten Triumeq tablets were ground into fine powder, the powder equivalent to 1.25 tablets was weighed into a 100 mL flask, and 50% acetonitrile was added to volume. The solution was sonicated for 30 min, and then centrifuged for 20 min at 8,000 rpm. After centrifugation, the supernatant was transferred to tubes for the next step.

Sample solution for method screening and optimization

The related compounds were spiked into the Triumeq drug product solution at a 10% concentration level relative to APIs for screening and at a 0.1% concentration level for optimization.

Working solutions for the standard curve

Working solutions for the standard curve were prepared to concentrations shown in Table 2.

Table 1. Abacavir, lamivudine, dolutegravir, and their related compounds reference standards

Standards	CAS	Vendor	P/N
Abacavir sulfate	188062-50-2	Macklin	A833205
Lamivudine	134678-17-4	Damas-beta	21280A
Dolutegravir sodium	1051375-19-9	Macklin	G872900
Cytosine	71-30-7	Macklin	C804556
Uracil	66-22-8	Macklin	U820313
Salicylic acid	69-72-7	Macklin	S817529
Cyclopropyl diaminopurine abacavir	120503-69-7	Macklin	N892752
Lamivudine impurity I	173829-09-9	China National Institutes for Food and Drug Control (NIFDC)-National Drug Reference Standards	101317
Lamivudine impurity III	145986-07-8	NIFDC	101318
Lamivudine impurity V	160552-54-5	NIFDC	101319
Abacavir related compound A	124752-25-6	Sigma-Aldrich	PHR2064
Abacavir related compound B	141271-12-7	Sigma-Aldrich	PHR2065
Abacavir related compound C	172015-79-1	Sigma-Aldrich	PHR2066
Abacavir related compound D	1443421-69-9	USP	R152H0

Table 2. Working solution of abacavir sulfate, lamivudine, dolutegravir sodium

	Concentration of working solutions (mg/L)						
Compounds	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7
Abacavir sulfate	0.4	1.0	5.0	25.0	50.0	100.0	500.0
Lamivudine	0.2	0.5	2.5	12.5	25.0	50.0	250.0
Dolutegravir sodium	0.03	0.07	0.4	2.1	4.2	8.3	41.7

Results and discussion

A systematic method development protocol consists of a series of steps, which typically includes defining the sample preparation and separation criteria, method scouting/screening, and method optimization. Each step has a clear objective and enables the analyst to evaluate the key parameters affecting selectivity and resolution one by one, and then make an adjustment based on his or her knowledge, therefore providing a reproducible and cost-effective approach.

The criteria for this method are listed in the following:

- The method should separate all analytes with a USP resolution ≥2.0.
- Peaks should have reasonable peak shape; the USP peak asymmetry should be within 0.7–1.3.
- MS-compatible eluents should be used to reduce any further method changes for MS.

Method screening

In the screening stage, the main objective is to identify an appropriate column and eluent conditions to achieve a suitable chromatographic profile.

Columns, gradient, and eluents used for method screening are listed in Table 3. Five columns with different ligands and carbon loading were selected that cover a wide selectivity and pH range. All the columns were performed at pH 2.7 and 6.6 with methanol and acetonitrile first. For a streamlined approach to column screening, Automated Method Scouting can be employed to further improve the laboratory workflow.[7]

Peak number, resolution, and peak shape are used to evaluate the performance of the conditions. The best separation performance was achieved by using an Acclaim 120 C18 column, pH 2.7 in the first screening. For the organic phase, the retention time of the last peak is shorter when using acetonitrile due to its greater elution strength. Compared with acetonitrile, methanol provides a better separation for most analytes and with stronger retention for cytosine, which can avoid the influence of the solvent peak. So, methanol was chosen as a strong solvent in the following experiments.

Table 3. Columns, gradients, and eluents used for method screening

Columns			
Thermo Scientific™ Acclaim™ 120 C18, 3 µm, 4.6 × 150 mm (P/N 059133)			
Thermo Scientific™ Acclaim™ Polar Advantage II C18, 3 µm, 4.6 × 150 mm (P/N 063191)			
Thermo Scientific™ Hypersil GOLD™ C18, 3 µm, 4.6 × 150 mm (P/N 25003-154630)			
Thermo Scientific™ Hypersil GOLD™ C18 aQ, 3 µm, 4.6 × 150 mm (P/N 25303-154630)			
Thermo Scientific™ Hypersil GOLD™ PFP, 3 µm, 4.6 × 150 mm (P/N 25403-154630)			
Aqueous eluent			
0.1% formic acid in water, pH 2.7			
20 mM ammonium formate, pH 6.6			
0.02% formic acid and 10 mM ammonium formate in water, pH 3.9			
0.002% formic acid and 10 mM ammonium formate in water, pH 5.0			
10 mM ammonium formate in water, pH 6.0			
Organic eluent			
Methanol			
Acetonitrile			
Methanol with 0.01% formic acid			
Gradient			
Time (min)	*Flow rate (mL/min)*	*Aqueous eluent (%)*	*Organic eluent (%)*
0	1.0	95	5
5	1.0	95	5
15	1.0	5	95
17	1.0	5	95
17.1	1.0	95	5
22	1.0	95	5

Chromatograms (a) and (e) in Figure 2 show the results produced by using methanol at pH 2.7 and 6.6 on an Acclaim 120 C18 column. Peaks were identified by using the peak area and UV spectrum. Dramatic changes in retention time, peak order, and peak shape between the low and high pH were observed. Often, pH change can have a significant impact on the retention of ionizable compounds, such as salicylic acid, lamivudine impurity I, and abacavir related compound D in this set of compounds.

To get a better chromatographic profile, further separations at pH 3.9, 5.0, and 6.0 were investigated. Methanol with 0.01% formic acid was also explored to reduce the peak tailing of dolutegravir. The results show that the best performance relative to resolution, peak tailing, and peak shape was achieved by using pH=5 aqueous phase and methanol with 0.01% formic acid (Figure 2).

Method optimization

The optimization phase is usually the most time-consuming step in method development. In this phase, the method was further optimized to achieve the best separation between the chosen pair of critical peaks. The concentration of related compounds was reduced from 10% of the APIs to the target concentration of 0.1%, which makes the separation more challenging.

To improve the resolution between salicylic acid and cyclopropyl diaminopurine abacavir, the gradient was adjusted. A shallow gradient will usually increase the resolution and cause a decrease of sensitivity. We tried a shallower gradient by decreasing the percentage of methanol (with 0.01% formic acid) at the gradient endpoint from 95% to 80%, and then to 60%.

By using 60% methanol (with 0.01% formic acid), the resolution between salicylic acid and cyclopropyl diaminopurine abacavir was improved to 1.82 (Figure 3). Under this condition, we found that there were coeluting compounds with abacavir. Traditionally, the fast, broad, linear gradient used in the method screening phase does not provide enough resolution near the API for closely eluted impurities.[4,5] To separate the coeluting impurities, a multi-segment gradient was used as shown in Figure 4.

An isocratic hold of 5 minutes at 95% aqueous phase was utilized to retain the polar compounds. This was followed by a shallow gradient in 20 min from 10% to 40% methanol (with 0.01% formic acid), which maximized the resolution around the API peak. At the end of this step, abacavir and the coeluting compounds were well separated with a resolution of 4.73 (Figures 3 and 4), and the peak purity of abacavir was improved from 965 to 997. A steep gradient segment and hold for 2 minutes with 90% of methanol was used to elute highly retained components.

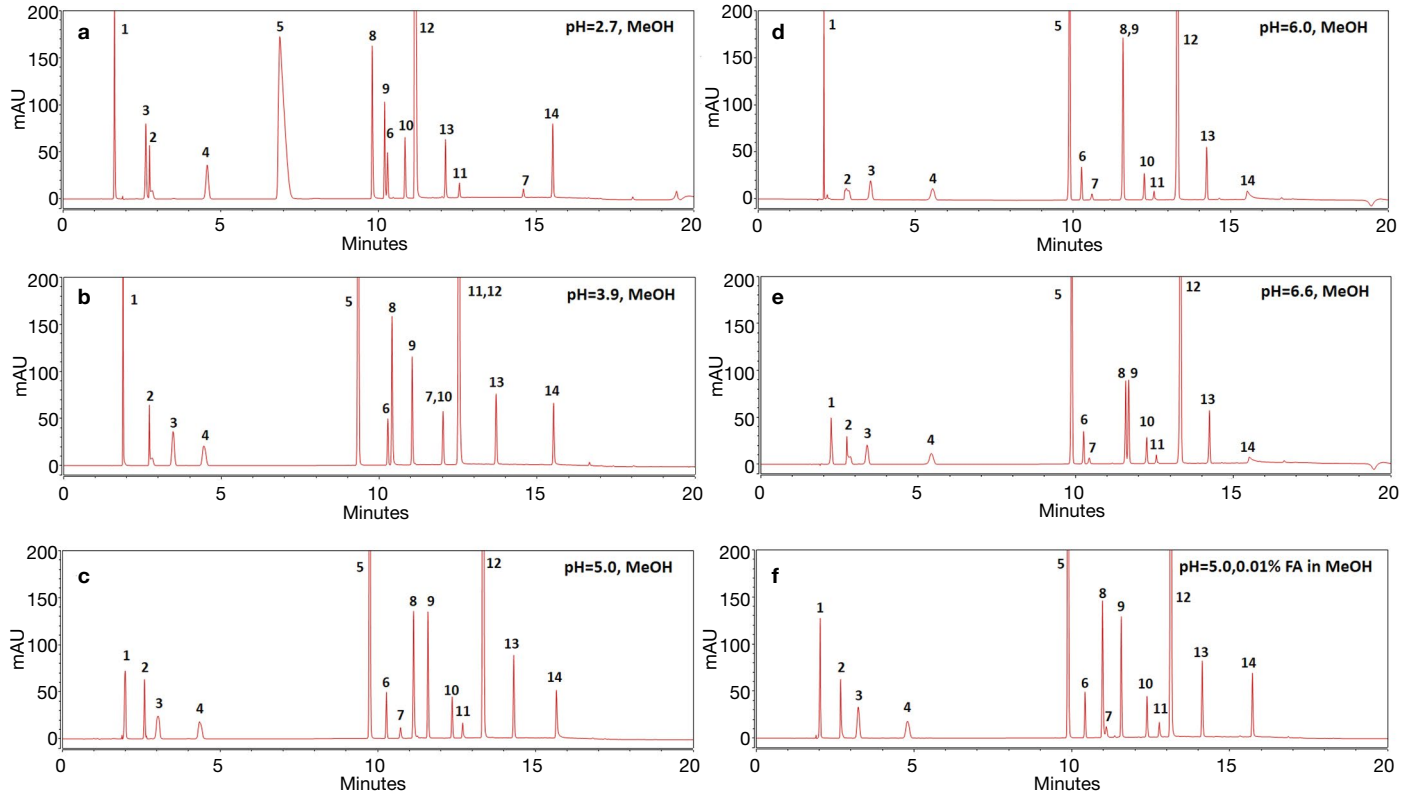


Figure 2. Example chromatograms with different pH on an Acclaim 120 C18 column. Peak ID: 1. Cytosine; 2. Uracil; 3. Lamivudine impurity I; 4. Lamivudine Impurity V; 5. Lamivudine; 6. Lamivudine Impurity III; 7. Salicylic acid; 8. Cyclopropyl diaminopurine abacavir; 9. Abacavir related compound A; 10. Abacavir related compound B; 11. Abacavir related compound C; 12. Abacavir; 13. Abacavir related compound D; and 14. Dolutegravir

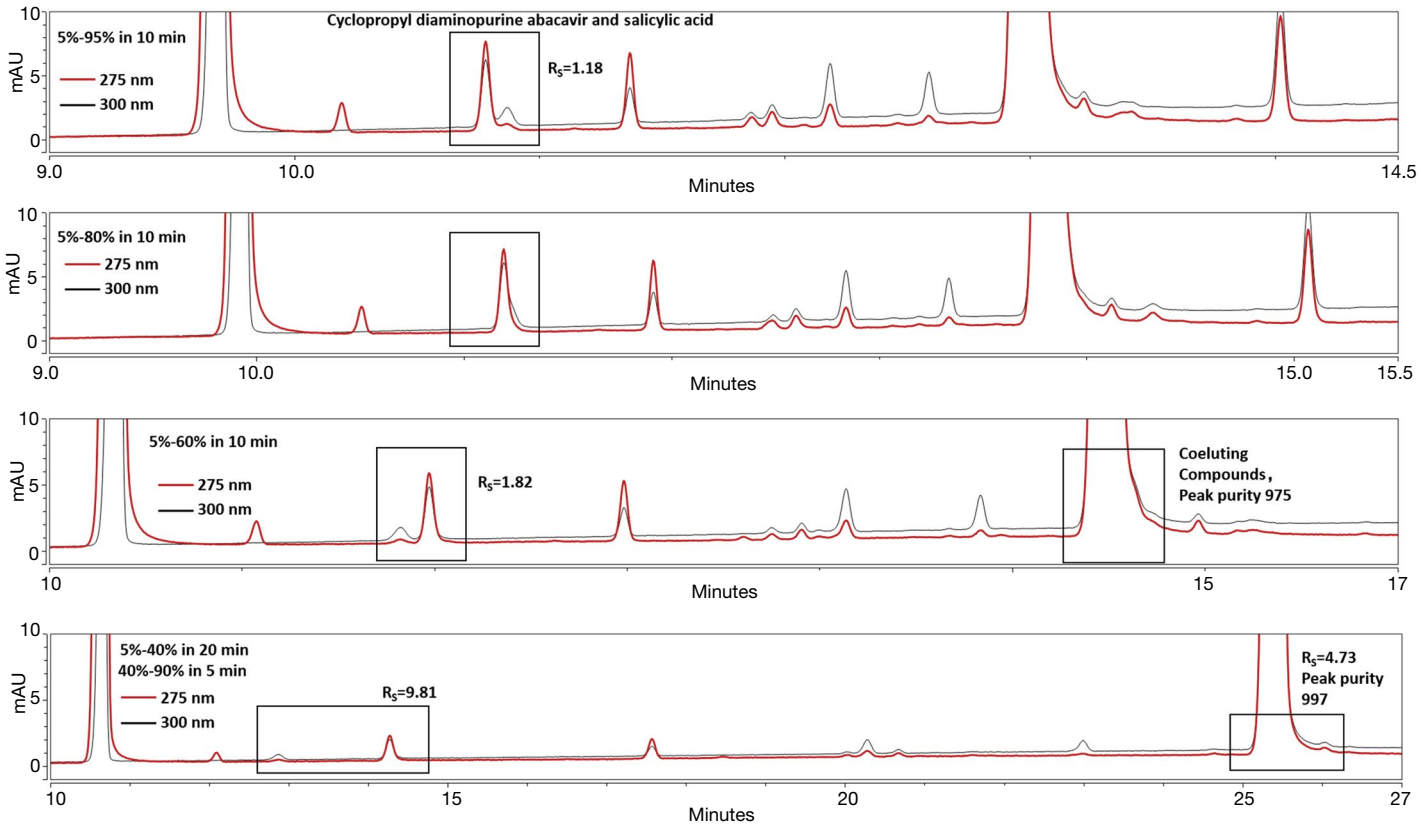


Figure 3. Optimization of the gradient using methanol (with 0.01% formic acid) on an Acclaim 120 C18 column

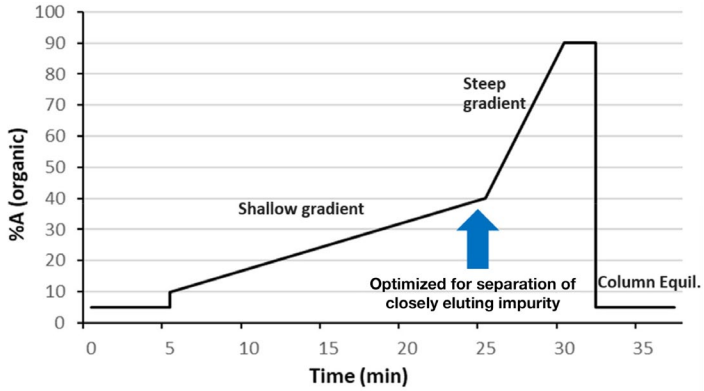


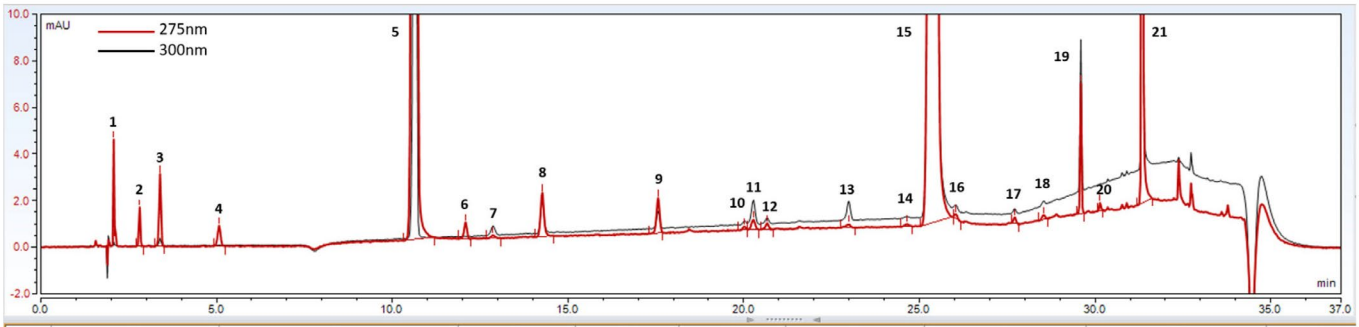
Figure 4. Multi-segment gradient used for separation

Final HPLC method

The final HPLC method conditions are shown in Table 4. The results generated by this method (Figure 5) were compared with the performance criteria that were set at the initial step. All analytes have a USP resolution >2.0 and peak tailing within 0.7—1.3. The eluent is compatible with mass spectrometric detection, which enables a straightforward transfer to further analysis with mass spectrometry for the structural elucidation of unknown impurities.

Table 4. Final HPLC method conditions

Parameter	Setting		
Column	Acclaim 120 C18, 3 µm, 4.6 × 150 mm		
Mobile phase	A: Methanol with 0.01% formic acid B: 10 mM ammonium formate, 0.002% formic acid in water		
Gradient	*Time (min)*	*Mobile phase A (%)*	*Mobile phase B (%)*
	0	5	95
	5	5	95
	5.1	10	90
	25	40	60
	30	90	10
	32	90	10
	32.1	5	95
	37	5	95
Flow rate	1.0 mL/min		
Column temp.	30 °C		
Sample compartment temp.	4 °C		
Injection volume	20 µL		
Needle wash solvent	50% methanol		
Autosampler wash mode	After draw		
Detector	275 nm, 300 nm, 20 Hz, 0.2 response time, 4 nm bandwidth 3D Field: 190—600 nm, bandwidth, 4 nm		



	A	B	C	D	E	F	G	H	I
1	Peak No.	Peak Name	Ret.Time min	Rel.Area %	Area mAU*min	Width (50%) min	Asymmetry (EP)	Resolution (USP)	
4	1	Cytosine	2.072	0.04	0.0937	0.019	1.26	13.58	
5	2	Uracil	2.812	0.04	0.0867	0.047	0.99	6.48	
6	3	Lamivudine impurity I	3.392	0.09	0.2056	0.061	0.94	12.74	
7	4	Lamivudine impurity V	5.071	0.04	0.0890	0.090	0.94	37.51	
8	5	Lamivudine	10.652	37.16	81.9455	0.081	0.84	10.32	
9	6	Lamivudine impurity III	12.092	0.03	0.0629	0.083	0.86	5.83	
10	7	Salicylic acid	12.862	0.01	0.0184	0.104	1.05	9.81	
11	8	Cyclopropyl diaminopurine abacavir	14.272	0.10	0.2152	0.099	1.12	19.89	
12	9	Abacavir related compound A	17.567	0.07	0.1589	0.096	0.94	22.88	
13	10	Unknown compound	20.021	0.00	0.0102	0.092	0.85	2.41	
14	11	Abacavir related compound B	20.283	0.02	0.0494	0.112	0.90	3.27	
15	12	Unknown compound	20.672	0.01	0.0287	0.096	0.81	48.62	
16	13	Abacavir related compound C	22.983	0.01	0.0206	0.098	0.78	48.21	
17	14	Unknown compound	24.651	0.01	0.0139	0.069	1.06	6.25	
18	15	Abacavir	25.428	60.79	134.0568	0.121	0.79	4.73	
19	16	Unknown compound	26.026	0.01	0.0158	0.087	1.30	25.94	
20	17	Unknown compound	27.702	0.01	0.0200	0.079	1.10	6.78	
21	18	Unknown compound	28.529	0.01	0.0186	0.085	1.03	9.19	
22	19	Abacavir related compound D	29.596	0.12	0.2544	0.041	0.99	7.86	
23	20	Unknown compound	30.137	0.01	0.0197	0.053	1.18	18.87	
24	21	Dolutegravir	31.342	1.43	3.1579	0.035	1.09	n.a.	
25	Maximum			60.79	134.0568	0.121	1.30	48.62	
26	Minimum			0.00	0.0102	0.019	0.78	2.41	
27	Sum			100.00	220.5418				

Figure 5. Final separation results of the abacavir, lamivudine, dolutegravir, and related compounds at the 0.1% level of APIs from Chromeleon CDS

Method validation

The developed method was validated according to ICH Q2 (R1) guidelines[6] with respect to specificity, linearity, accuracy, precision, limit of detection (LOD) and limit of quantitation (LOQ), and robustness.

Specificity

The blank, reference standards, drug product solutions, and drug product solution spiked with related compounds were injected to demonstrate the specificity. It shows that there is no interference to the APIs and related compounds from unknown impurities and excipients (Figure 6). The peak purity match values of APIs were also checked; all the values are higher than 990.

Linearity and range

The linearity was established by injecting at least five concentration levels that cover the 80% to 120% of the drug test concentration. The results are shown in Table 5, all with correlation coefficients (R) higher than 0.999.

Accuracy

Accuracy was evaluated by preparing triplicate drug product solutions spiked with abacavir, lamivudine, and dolutegravir at three levels (LOQ, 100% (assay level), and 150%); each sample was injected twice. Results are shown in Table 5. The recovery ranged from 88% to 96% with an RSD <10% for LOQ level and from 95% to 104% with an RSD <5% for assay level and 150% level; all pass the acceptance criteria.

Precision

Repeatability was assessed by preparing six spiked samples at 100% assay level and low level. Intermediate precision was evaluated by preparing six spiked solutions by different analysts on different days, then injecting on a second HPLC system (Vanquish Core). As shown in Table 5, the RSD ranged from 0.4% to 1.4% for assay level, from 1.4% to 3.4% for low level, and from 0.6% to 3.2% for intermediate precision; all met our acceptance criteria.

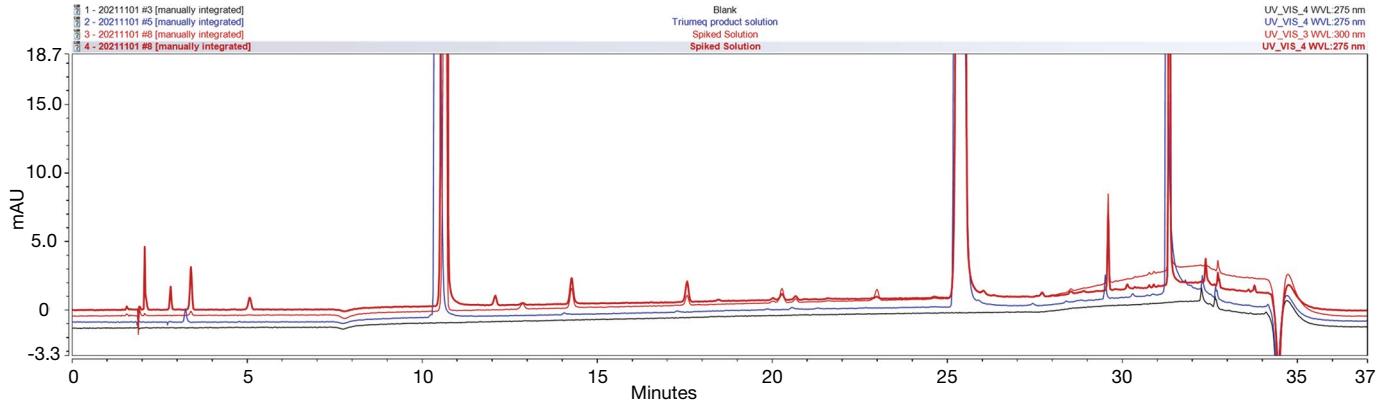


Figure 6. The overlaid chromatogram of blank, drug product solution, and spiked solution

Table 5. Validation results of abacavir, lamivudine and dolutegravir

Validation element	Acceptance criteria	Results		
		Abacavir	Lamivudine	Dolutegravir
Specificity	No significant interference from unknown impurities and excipients. Resolution ≥ 1.5	No significant interference. Resolution >2.0		
Linearity				
Linearity correlation coefficients	R ≥0.999	R=1.000	R=1.000	R=0.999
Linearity range	mg/L	0.40–500	0.20–250	0.033–41.65
Accuracy				
LOQ level	85–115%	87.95	96.21	88.86
Assay levels	95–105%	97.51	99.99	103.99
150% levels	95–105%	101.29	98.00	95.45
Precision				
Repeatability at assay level	RSD ≤2%	0.39	0.60	1.39
Repeatability at low level	RSD ≤5%	1.63	1.41	3.41
Intermediate precision	RSD ≤5%	2.27	0.61	3.18
LOQ	mg/L	0.40	0.20	0.033
LOD	mg/L	0.18	0.09	0.015

LOD and LOQ

LOD and LOQ for APIs and related compounds were obtained by injecting diluted solutions with a known concentration and calculating the signal to noise of 3:1 for LOD and 10:1 for LOQ.

The concentration of abacavir is 2 times that of lamivudine and 12 times that of dolutegravir in Triumeq drug tablet. The same ratio was used to prepare the working solution, LOD solution, and LOQ solution. The UV absorbance of dolutegravir at 275 nm is lower than abacavir and lamivudine at the same level, so LOD and LOQ was determined by dolutegravir. The LOQ and LOD results are shown in Table 5 and Table 6; the LOD of dolutegravir and related compounds can be less than 0.05 ppm due to the high sensitivity of the Vanquish Core DAD.

Table 6. LOD and LOQ for the related compounds

Related compounds	Results	
	LOD (mg/L)	LOQ (mg/L)
Cytosine	0.01	0.04
Uracil	0.02	0.04
Lamivudine impurity I	0.02	0.04
Lamivudine impurity V	0.02	0.08
Lamivudine impurity III	0.04	0.08
Salicylic acid	0.04	0.20
Cyclopropyl diaminopurine Abacavir	0.02	0.08
Abacavir related compound A	0.02	0.08
Abacavir related compound B	0.02	0.08
Abacavir related compound C	0.02	0.08
Abacavir related compound D	0.01	0.05

Robustness

Small deliberate variations were made to the HPLC conditions to evaluate the robustness, including the column temperature (30 ± 5 °C), flow rate (1.0 ± 0.1 mL/min), pH of the buffer (5.0 ± 0.3), and columns with different batches. Under these conditions, all analytes had a USP resolution ≥1.5 and peak tailing within 0.7–1.3, which demonstrates the robustness of this method.

Conclusion

A reversed-phase HPLC method was developed for separation and determination of abacavir, lamivudine, dolutegravir, and their related compounds in Triumeq by using a Vanquish Core HPLC, an Acclaim 120 C18 column, Chromeleon CDS, and MS-compatible eluents. The demonstrated stepwise approach enables the analytical laboratory to be efficient and cost-effective in HPLC method development.

The final method provides an adequate separation for all analytes with a USP resolution ≥2.0 and peak tailing within 0.7 to 1.3. It has good linearity, precision, and accuracy and can be used for routine analysis of the drug Triumeq.

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