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Step-by-step optimization of the HILIC method for simultaneous determination of abacavir, lamivudine, and zidovudine from dosage form

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Abstract: Combined therapy for HIV treatment shows superior efficacy in comparison to single therapy. Furthermore, the separation and determination of the combined dosage forms hold a significant place in the pharmaceutical industry. Not only reverse phase high-performance liquid chromatography (HPLC) but also hydrophilic interaction liquid chromatography (HILIC) can be used for these purposes. Contrary to conventional RP systems, HILIC may be an alternative for the analysis of polar substances. The aim of this study was the step-by-step development of a simple, rapid, and reliable method for the simultaneous determination of antiviral compounds from their marketed formulation (Trizivir). In order to achieve this goal, various mobile phase systems, buffer types, and concentrations were prepared to provide an appropriate separation. Different types of columns were tested to find the best resolution and high efficiency for the studied compounds. The proposed method provided a simple procedure for the simultaneous analysis of abacavir, lamivudine, and zidovudine in their pharmaceutical preparation within approximately 2 min using the conventional HPLC system. The developed method was fully validated according to the International Council for Harmonisation guidelines from the viewpoint of selectivity, sensitivity, precision, accuracy, linearity, limit of detection, and quantification.

Key words: Abacavir, HILIC, HPLC, lamivudine, zidovudine

1. Introduction

Abacavir (ABC), lamivudine (LMV), and zidovudine (ZDV) (Figure 1) are all nucleoside reverse transcriptase inhibitors (NRTIs) and are potent selective inhibitors of HIV-1 and HIV-2. All 3 pharmaceutically active components are metabolized sequentially by intracellular kinases to the respective 5'-triphosphate. The combination of ABC, LMV, and ZDV is also very useful for pregnant women to decrease the risk of transmission from mother to child. ABC and LMV are rapidly absorbed following oral administration, with a bioavailability of about 80%. On the other hand, ZDV is absorbed rapidly from the gastrointestinal tract, with a bioavailability of approximately 60%–70%. Finally, the combination of these active components helps to reduce HIV's resistance to the drugs individually due to tough mutation [1,2].

To date, there have been some publications describing analytical methods for the simultaneous determination of ABC, LMV, and ZDV in pharmaceutical dosage forms and biological samples in addition to other HIV compounds using liquid chromatography (LC) tandem-mass spectrometry [3,4]. These hyphenated techniques may provide high selectivity of the assay, but they may also cause also some disadvantages of operational complexity and high cost, which hamper their further application. In contrast to these methods, high-performance

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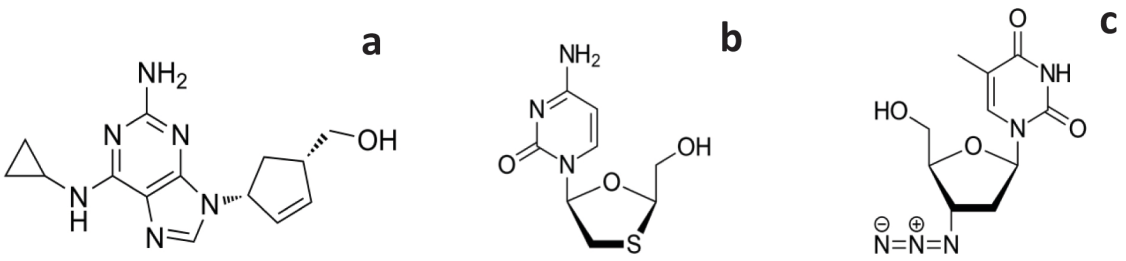

Figure 1. Chemical structures of ABC (a), LMV (b), and ZDV (c).

LC (HPLC) with ultraviolet (UV) detection offers basic instrumentation and needs a less experienced analyst for the separation of this ternary mixture [5,6]. A literature survey revealed that all of the methods were performed under reverse phase (RP) conditions and many of them required a long analysis time, except for a study by Djurdjevic et al. [6]. In this case, the authors used chemometric techniques for the optimization of their methodology. However, they did not check the stability-indicating capability of the developed method. Based on the literature, there has not been any single method indicating the usage of hydrophilic interaction LC (HILIC) for the simultaneous separation and quantification of ABC, LMV, and ZDV.

From this perspective, HILIC provides an alternative approach to effectively separate polar substances. It has also been reported that HILIC is a combination of normal phase, RP, and, ion exchange chromatography. However, in comparison to the normal phase and ion exchange chromatographic techniques, the separation mechanism used in HILIC is more complicated than described in the literature. Retention can be explained by the partitioning of analytes between the mobile phase and a water-enriched layer. If the hydrophilicity of the analyte is higher, the partitioning equilibrium of the analyte shifts towards the immobilized water layer in the stationary phase, and thus, there are more analytes to be retained [2]. Charged analytes are more hydrophilic than their uncharged forms, so they are retained more in HILIC [7,8]. Thus, the formation of electrostatic interactions with charged analytes can be caused by the existence of ionized groups or underivatized ionized silanol groups in the stationary phase [7–11]. Whether these interactions are attractive depends on both the analyte and the charged state of the stationary phase. The increase in retention times is caused by electrostatic attractions, which are between positively charged compounds and negatively charged stationary phases, and it can be the opposite. In HILIC, the possibility of direct interactions, i.e. hydrogen bonds, of the analyte with the stationary phase in addition to partitioning into the water layer has been debated for a long time [7]. In the literature, some research has investigated the factors that affected retention in chromatographic systems using the linear solvation energy relationship model, which was supported by solvatochromic descriptors, including the acidity, basicity, and hydrogen bonding parameters provided. Interaction through the hydrogen bond with the stationary phase can be formed by molecules with hydrogen donor or hydrogen acceptor functionalities as proved by the high values found for these parameters [7].

Despite its complex mechanism, HILIC has the following advantages over RP-LC: retention of polar compounds, better peak shapes for bases, higher flow rates with low background pressures, and enhanced mass spectrometer sensitivity due to the high organic content in the mobile phase. Similar to the normal phase, retention increases with increased polarity of the analyzed compounds and/or decreased mobile phase polarity. However, the normal phase LC drawback of insolubility of hydrophilic compounds is largely solved under HILIC conditions, because of its mobile phase properties. A fraction of the mobile phase becomes an integral part of the stationary phase, because a water-enriched liquid layer is established within the stationary phase. For this

reason, at least 2% of water is needed in the mobile phase to sufficiently hydrate the stationary phase [12,13].

Within the frame of the explanations above, the aim of this research was the step-by-step optimization and development of a simple, rapid, fully validated stability-indicating HILIC method with acceptable detection limits for the simultaneous determination of ABC, LMV, and ZDV. Furthermore, the applicability of the developed method is demonstrated by analyzing a pharmaceutical preparation of this ternary mixture.

2. Results and discussion

2.1. Step-by-step development of the chromatographic method

Since the compounds are polar, HILIC is an alternative for the separation and determination of this ternary mixture. It is very well known that the column chemistry, mobile phase composition, mobile phase ratio, buffer type and concentration, pH, flow rate, and temperature of the column can affect the retention mechanism as well as the selectivity [14,15]. For this reason, each step for the development of this methodology was demonstrated in detail.

2.1.1. Optimization of stationary phase

In the present work, initial steps were performed by arranging the most suitable analytical column. For this reason, the Kinetex HILIC (150 mm ×4.6 mm, 2.6 µm), Kinetex HILIC (150 mm ×4.6 mm, 5 µm) (Phenomenex, Torrance, CA, USA), XBridge HILIC (150 mm ×4.6 mm, 2.5 µm) (Waters, Milford, MA, USA), and ZIC HILIC (250 mm ×4.6 mm, 5 µm) (Merck KGaA, Darmstadt, Germany) columns were used to find optimal separation results (Figures 2a–2d). The aim was to compare 4 different columns based on their length, particle size, and particle type and obtain the shortest analysis time, sharper peak shapes, and efficient separation. Based on the results, which are illustrated in Figure 2 and tabulated in Table 1, a successful separation was achieved using Kinetex HILIC columns. The superiority of these columns comes from superficially porous particles. The benefits of these materials have been mentioned in several publications [14–17]. When comparing the particle sizes, the estimation of smaller particles increased the efficiency. The only advantage of 5-µm particles is low back pressure. However, under HILIC conditions, due to the high organic content, the pressure value was within the acceptable limits. For this reason, a Kinetex HILIC column with 2.6-µm particles was selected in the current study for the simultaneous determination of ABC, LMV, and ZDV.

2.1.2. Optimization of mobile phase

In order to evaluate the behavior of the ternary mixture under HILIC conditions, a mobile phase mixture was selected with acetonitrile (ACN), ammonium acetate or ACN, or ammonium formate buffers, in which successful baseline separation was achieved. As a starting point, the effect of the ACN was evaluated. For this reason, the amount of ACN varied in the range of 80%–98% (v/v) with 20 mM ammonium acetate as the buffer component. Related chromatograms are illustrated in Figure S1 and the results are given in Table S1.

According to the chromatograms, the shortest analysis time was achieved using the composition of ACN:buffer (20 mM ammonium acetate) (80:20, v/v). Contrary to this phenomenon, the separation efficiency was lost between the critical peak pairs of LMV and ABC. Furthermore, it is known that in HILIC, the concentration of the buffer may cause separation between the critical peak pairs, which is why the effect of this parameter was evaluated herein (Figure S2 and Table S2). Thus, the molarity of the buffer solution changed from 5 mM to 100 mM and the best separation with a suitable analysis time was achieved using 100 mM as the buffer concentration (Figure S2), and the results are demonstrated in Table S2 in detail.

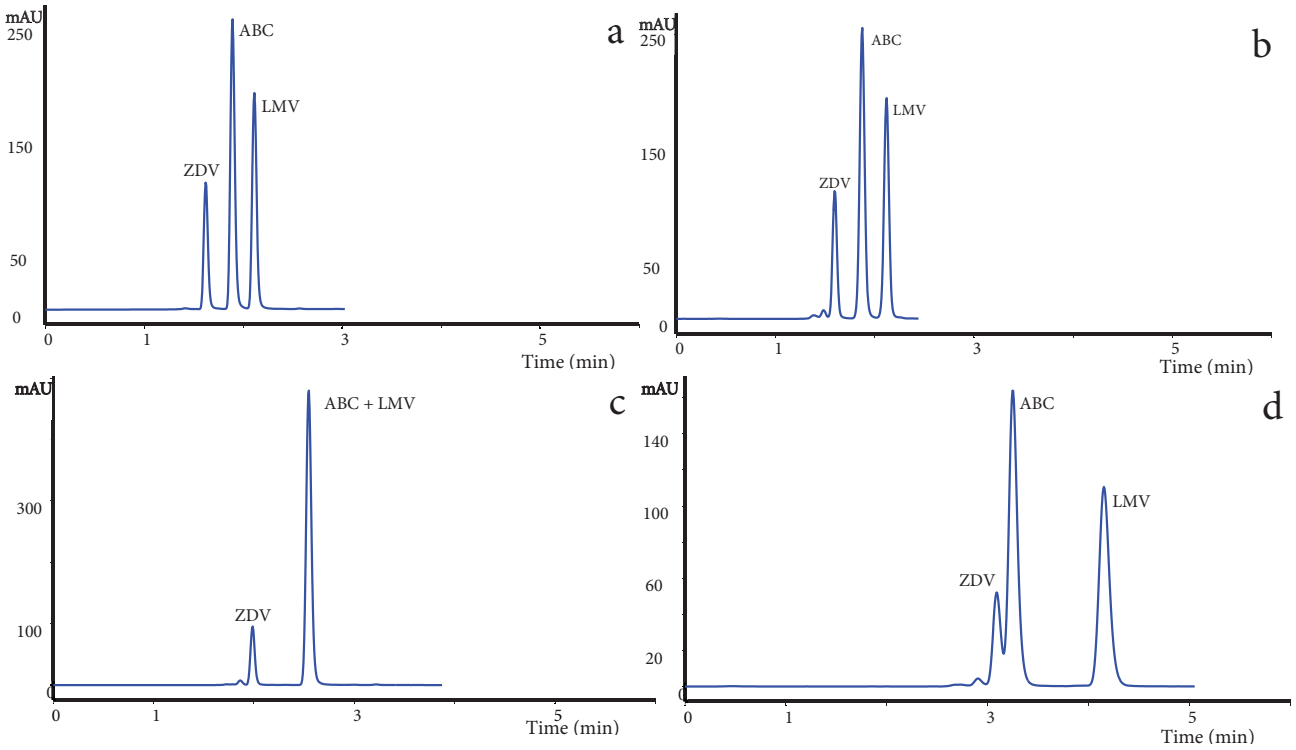

Figure 2. Kinetex HILIC (150 mm ×4.6 mm ×2.6 µm) column (a), Kinetex HILIC (150 mm ×4.6 mm ×5 µm) column (b), XBridge HILIC (150 mm ×4.6 mm ×2.5 µm) column (c), ZIC HILIC (250 mm ×4.6 mm ×5 µm) column (d), 25 °C, flow rate: 1 mL min^{-1}, pH 6.68, wavelength: 280 nm, and mobile phase: ACN:buffer solution-100 mM ammonium acetate (v/v) (80:20).

Table 1. Comparison of the columns [25 °C, flow rate: 1 mL min^{-1}, pH 6.68, mobile phase: ACN:buffer solution-100 mM ammonium acetate (v/v) (80:20)].

Column	Pressure (bar)	ZDV				ABC				LMV			
		t_R (min)	N	R_S	α	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α
Kinetex HILIC (150 mm ×4.6 mm ×2.6 µm)	108	1.65	6548	-	-	2.00	7959	3.26	2.46	2.10	9921	2.61	1.48
Kinetex HILIC (150 mm ×4.6 mm ×5 µm)	52	1.59	5977	-	-	1.87	6359	3.12	2.39	2.12	8489	2.63	1.51
XBridge HILIC (150 mm ×4.6 mm ×2.5 µm)	110	1.98	9456	-	-	-	-	-	-	-	-	-	-
ZIC HILIC (250 mm ×4.6 mm ×5 µm)	89	3.09	6964	-	-	3.25	7010	1.05	1.44	4.15	7813	5.24	2.73

Another mobile phase parameter examined in this study was the buffer type and its pH. The change of mobile phase pH at about ±1 unit had no significant effect on the retention mechanism. The related chromatograms and table are given as Supplementary material (Table S3 and Figure S3). On the other hand, the type of buffer was changed and ammonium formate was used instead of ammonium acetate. Not only the

resolution between the critical peak pairs but also the efficient plate numbers of the compounds were decreased slightly. Due to the results, which are demonstrated in Figure S4 and Table S4, ammonium acetate was used in all of the procedures.

2.1.3. Optimization of the temperature and flow rate

The column oven temperature is an important parameter in LC. The temperature was varied between 25 and 45 °C to demonstrate the effect of the temperature. Figure S5 and Table S5 illustrated that there is no remarkable variation achieved within the different temperatures. Hence, 25 °C was used for the analysis to keep the column safe and avoid any possible damage due to the higher temperatures.

The flow rate of the system was varied from 0.5 mL min^{-1} to 2 mL min^{-1}. As in the literature, it was clear that the superficially porous silica particles reduced the back pressure in comparison with the totally porous silica packing materials, and it resulted in higher flow rates [18,19]. The flow rates that resulted in the present work are tabulated in Table S6 and the related chromatograms are illustrated in Figure S6. As can be clearly seen from the results, with the increased flow rate, efficient plate number and resolution were reduced. In the meantime, analysis time was also decreased. The results also demonstrated that it was possible to analyze this ternary mixture in 1 min by HPLC. However, it is hard to use these extreme conditions if selectivity is needed for both the biological matrix and the analysis of impurities. For this particular reason, 1 mL min^{-1} was selected as the optimum flow rate. As a result of the optimization study, final optimized conditions were determined as mentioned in Section 3.3.

2.2. Validation of the proposed method

The developed method was validated for specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, etc. System suitability was verified by determining 5 repeated injections of a standard solution of ternary mixture. RSD% of the retention time, resolution, theoretical plate number, selectivity, and tailing factor were also evaluated and determined. The system suitability test results are illustrated as Supplementary material in Table S7. These results demonstrated that the developed method was suitable for the validation and determination of the ternary mixture of ABC, LMV, and ZDV. The selectivity and specificity of the developed method was established by determining the purity of the peaks of the ternary mixture subjected to stressed conditions, as described in Section 3.4.3. In this study, Trizivir tablet formulation was prepared by including 50 µg mL^{-1} LMV and a certain amount of ABC and ZDV. As a result of these experiments, the developed method had enough selectivity for the validation and determination of the dosage forms, in addition to the possible degradation products (Figure 3). Furthermore, the degradation amount for each compound is demonstrated in Figure S7 as graphs. Based on these graphs, placing the solutions of the active pharmaceuticals ingredients in an 80 °C hot water bath caused only 3%–4% degradation, while the solid form degradation process caused 2% degradation for ZDV and about 8%–10% degradation for ABC and LMV. UV light treatment caused a small amount of degradation. Acid hydrolysis affected the LMV more than the ABC and ZDV. On the other hand, basic hydrolysis treatment with 0.1 M NaOH caused the most degradation when compared to the other conditions. Specifically, LMV and ZDV degraded 40% in 1 h. Finally, the oxidative treatment mostly affected the LMV and the degradation percentage was about 12%.

The validation results are given in Tables 2 and 3. All of the results demonstrated that the method was specific to 3 compounds separated from each other and no interfering peak appeared at the retention time of the related peak signals. Linearity over the range of concentrations between 0.5 and 100 µg mL^{-1} (n = 8) were

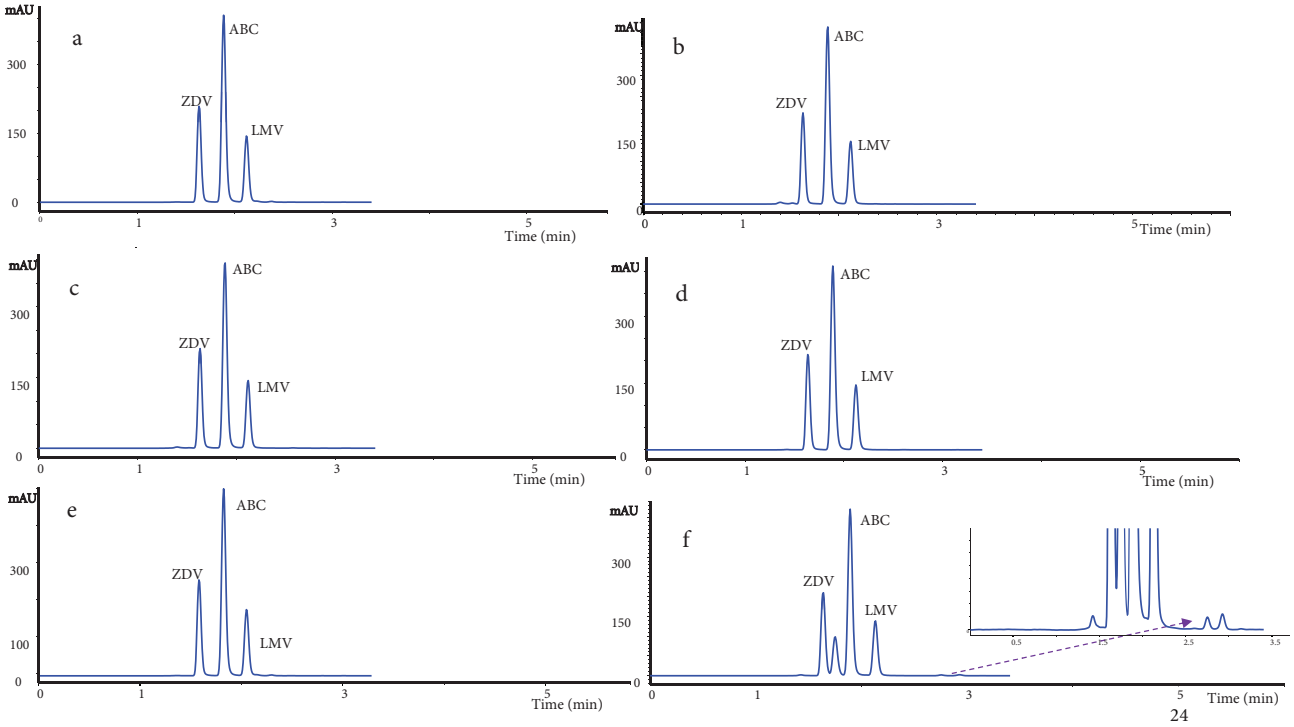

Figure 3. Conditions of degradation: waiting for 3 h in 80 °C hot water bath (a), waiting for 6 h under UV light (b), waiting for 12 h in 80 °C oven (c), acid hydrolysis with 0.1 N HCl for 12 h (d), base hydrolysis with 0.1 N NaOH for 12 h (e), and oxidation with 0.3% H_2O_2 for 12 h (f).

observed for ABC, LMV, and ZDV (Table 2 and Figure 4A). The linear regression data for the calibration curve demonstrated a good relationship in the ranges above. The calibration results, tabulated in Table 2, showed all of the necessary parameters. It showed good correlation and the determination coefficient was calculated as R^2>0.999. These results proved that the developed method was linear in the stated ranges. The LOD and LOQ were obtained and calculated from equations in which the standard deviation of response and the slope of the calibration curve were used [20]. Based on the calculations, the LOD values were 0.04 µg mL^{-1}, 0.03 µg mL^{-1}, and 0.02 µg mL^{-1} for ZDV, ABC, and LMV, respectively. Precision was tested in the frame of both intraday and interday evaluations. The low values of RSD% for method precision indicated that the method was reproducible (Table 2).

Accuracy of the developed method was tested by analyzing the pharmaceutical preparation and also via recovery experiments (Figure 4B). For this reason, the equations from the calibration curves were used for determination of the dosage form, namely Trizivir. The recovery experiments were performed by spiking the previously analyzed commercial formulation with a certain amount of standard solutions in a range between 25% and 150%. All of the experiments were repeated 5 times and the recovery, RSD%, and Bias% results are presented in Table 3. The mean of the spike recoveries was close to 100% at different levels, which indicated acceptable accuracy of the method. It also meant that the developed method was not affected by the possible interferences and excipients in the pharmaceutical formulation.

Table 2. Validation parameters and calibration results.

Validation parameters	ZDV	ABC	LMV
Linearity and range (µg mL^{-1})	0.5–100	0.5–100	0.5–100
Slope	12.86	22.70	17.31
Intercept	–4.04	1.65	–0.41
Correlation coefficient	0.999	0.999	0.999
SE of slope	0.045	0.09	0.062
SE of intercept	2.38	4.32	3.14
LOD (µg mL^{-1})	0.04	0.03	0.02
LOQ (µg mL^{-1})	0.12	0.09	0.08
Within-day precision RSD% *	1.15	0.92	0.77
Between-day precision RSD% *	0.83	1.17	0.65

*Each value is the mean of 5 experiments.

Table 3. Accuracy results of the simultaneous determination of ZDV, ABC, and LMV in dosage form.

	ZDV	ABC	LMV
Labeled amount (µg mL^{-1})	20.00	20.00	10.00
Amount found (µg mL^{-1})	19.84	19.50	10.10
RSD (%)*	0.31	0.24	0.42
Bias (%)	0.8	2.5	–1.0
Added (µg mL^{-1})	5	5	2.50
Found (µg mL^{-1})	4.9	5.09	2.46
Recovery (%)	98.0	101.8	98.4
RSD% of recovery *	0.46	0.79	0.83
Bias (%)	2.0	–1.8	1.6

*Each value is the mean of 5 experiments.

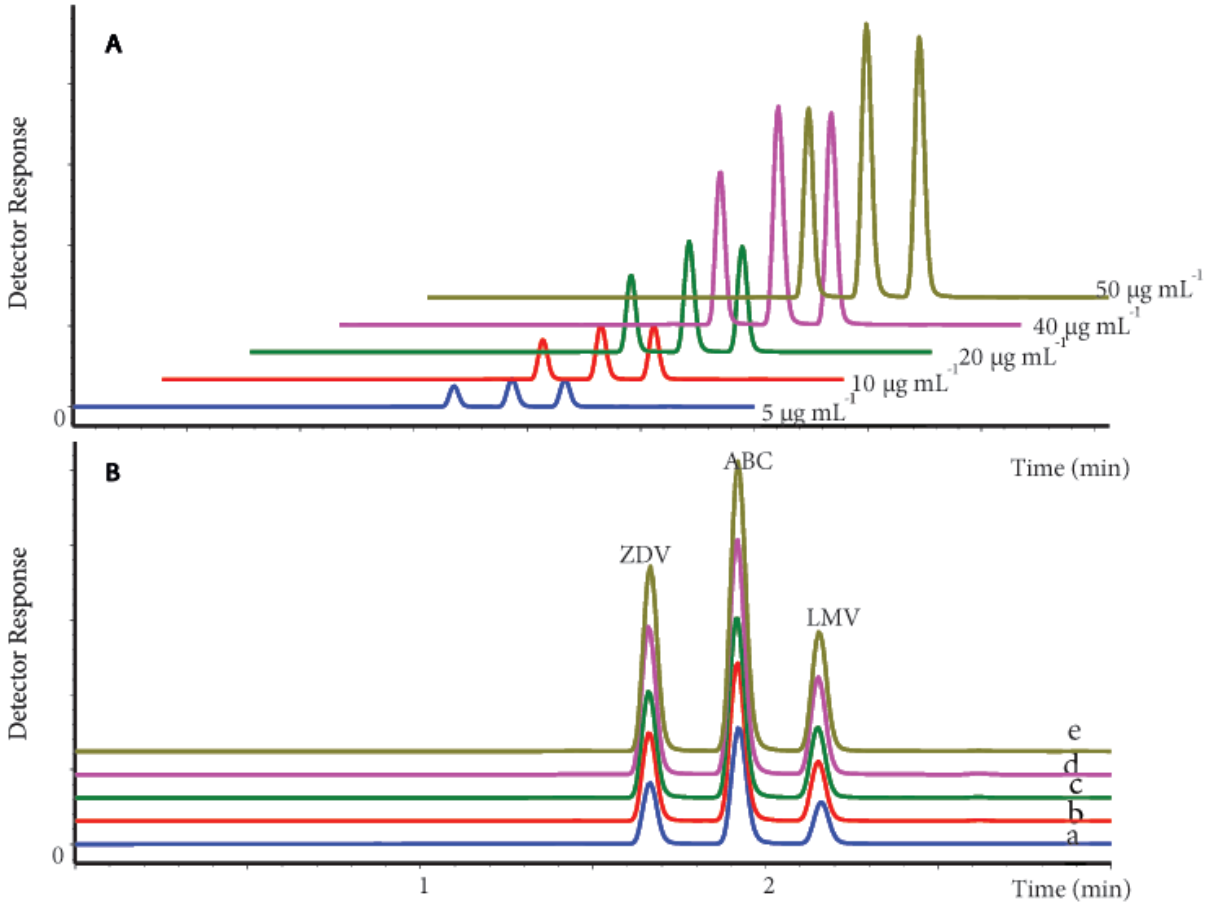

Figure 4. (A) Calibration results using optimized conditions ZDV, ABC, and LMV: 5 (a), 10 (b), 20 (c), 40 (d), and 50 (e) µg mL^{-1}. (B) Tablet sample under optimized conditions (a) and the addition of 25% (b), 50% (c), 100% (d), and 150% (e) active ingredient added into the tablet sample.

2.3. Conclusion

In summary, a systematic HPLC study for the simultaneous separation and determination of an ABC, LMV, and ZDV ternary mixture using HILIC was suggested for the first time herein. Based on the used mobile

phase, this method can be identified as MS-friendly. Since the run time was approximately 2 min, the suggested method was rapid, highly feasible, and reproducible and it can be used in research and development laboratories as well as quality control laboratories where economic and fast analysis are required. Furthermore, there are no sophisticated extraction and cleanup procedures applied in this methodology. From this point of view, it is an easy and applicable method. In addition, a step-by-step optimized HILIC method was developed and fully validated according to the International Council on Harmonisation (ICH) guidelines with respect to specificity, linearity, accuracy, precision, etc. Furthermore, the stability-indicating capability of the developed method was also demonstrated via stress degradation studies to underline the selectivity of the proposed technique. The analysis results illustrated that the developed method was much more efficient when compared with the previously suggested methods in the literature. On the other hand, it showed the advantage of using superficially porous silica particles as a stationary phase under HPLC or HILIC conditions.

3. Materials and methods

3.1. Instruments

The Agilent 1100 HPLC system (Agilent Technologies, Inc., Wilmington, DE, USA) consists of a G1313A ALS autosampler, G1316A temperature controller, G1311A quaternary pump, and 1315B DAD variable wavelength detector. This system was used for development of the HILIC method, stability-indicating experiments, and validation studies. Chromatographic grade water with conductivity lower than 0.05 µS cm^{-1} was obtained through a Milli-Q system (Millipore, Milford, MA, USA) and was used to prepare all necessary solutions. The pH of the buffer solutions was determined using a Thermo Scientific benchtop pH meter (Orion 3 Star Plus; Thermo Fisher Scientific Inc., Waltham, MA, USA).

3.2. Materials

The ABC and ZDV were kindly provided by Nobel İlaç San. ve Tic. A.S. (İstanbul, Turkey). The LMV and dosage forms (Trizivir) were kindly provided by GlaxoSmithKline (İstanbul, Turkey). All chemicals were of analytical grade and all solvents were of chromatographic grade. Toluene, methanol ACN, ammonium acetate, and ammonium formate were commercially available from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid, sodium hydroxide, orthophosphoric acid, and hydrogen peroxide were obtained from Merck (Darmstadt, Germany). As an analytical column, Kinetex HILIC (150 mm ×4.6 mm, 2.6-µm particle size) was purchased from Phenomenex Inc. (Torrance, CA, USA).

3.3. Chromatographic conditions

Optimization was carried out by a column oven temperature of 25 °C on a Kinetex HILIC (150 mm ×4.6 mm, 2.6 µm particle size) column. Before the first injection, the column was preconditioned at 25 °C for at least 25 min. The mobile phase, which was prepared daily and sonicated for 15 min before use, consisted of a mixture of ACN:ammonium acetate buffer (100 mM) solution (80:20, v/v) that was used for the separation and analysis of the ABC, LMV, and ZDV at a flow rate of 1 mL min^{-1} isocratically and an injected volume of 10 µL. Finally, UV detection was performed at 280 nm in all of the steps. In the present study, the dead volume was measured by injecting toluene solution (0.01% (v/v), in water).

3.4. Preparation of the solutions

3.4.1. Preparation of the stock solutions and calibration curves

Stock solutions of ABC, LMV, and ZDV (1000 µg mL^{-1}) were prepared in a mixture of ACN:water (80:20, v/v). All solutions were then kept in the dark in a refrigerator. Working solutions were prepared by the dilution of these stock solutions using the same solution. The concentrations of ABC, LMV, and ZDV were adjusted in the range of 0.5 to 100.0 µg mL^{-1}. The calibration graph for this method was created by plotting the peak area of the active compound against the drug concentration using 5 replicated analyses.

3.4.2. Preparation of the pharmaceutical dosage form

Five Trizivir tablets were weighed, which were claimed to contain 300 mg ABC, 300 mg ZDV, and 150 mg LMV. An accurate weight of the crushed and powdered combination equivalent to 1 tablet content was weighed and transferred to a 100-mL flask, diluted with a mixture of ACN:water (80:20, v/v), and ultrasonicated for about 10 min. Finally, the volume was completed to the limit with the same solvent. A syringe filter (0.45 µm) was used for filtration and the filtered stock solution (3000 µg mL^{-1}) was prepared in a calibrated flask.

3.4.3. Preparation of stress degradation samples for the selectivity studies

The ICH [20,21] guidelines recommend performing degradation studies for establishing the stability-indicating nature of the developed methodology. Degradation studies, including photolytic degradation at 254 nm, heating in an oven at 80 °C, acidic and basic hydrolysis, and oxidation, were performed to demonstrate the stability-indicating capability and selectivity of the proposed method using ABC, LMV, and ZDV, and their possible degradation products. The aliquot amount of powdered Trizivir tablets was weighed and put into an oven at 80 °C for thermal degradation, and the same dosage form was subjected to 254nm UV light for photolytic degradation studies. After treatment, 25mg of the bulk powders was dissolved in the same solvents that are described in the sections above and injected into the system. Furthermore, a constant amount of sample solutions was diluted with 0.1 M HCl for acidic degradation, 0.1 M NaOH for basic degradation, and 0.3% hydrogen peroxide for oxidation to achieve a final concentration of 50 µg mL^{-1}. Subsequently, all of the solutions were stirred for 5 min and left for 1 to 12 h under the described stress conditions.

3.5. Validation procedure

The validation procedure was carried out according to the ICH guidelines. The developed method was validated for quantitation of the ternary mixture of ABC, LMV, and ZDV from their raw formulations and marketed preparations from the viewpoint of system suitability, specificity, linearity, range, accuracy, precision, etc.

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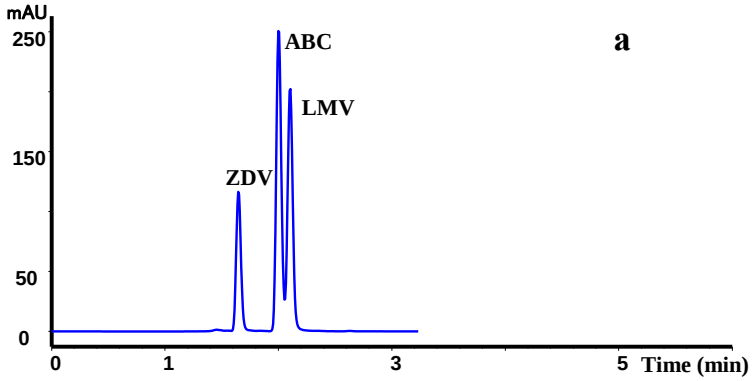
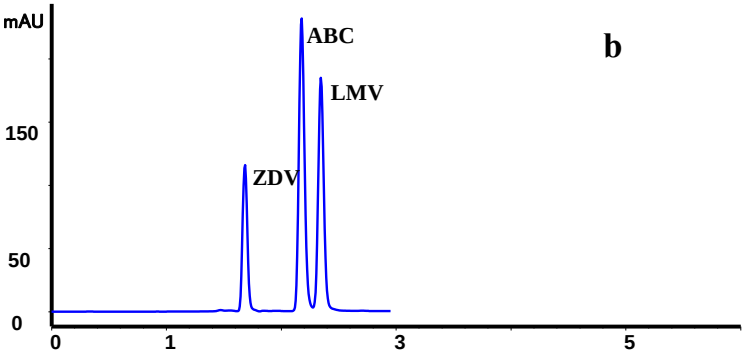
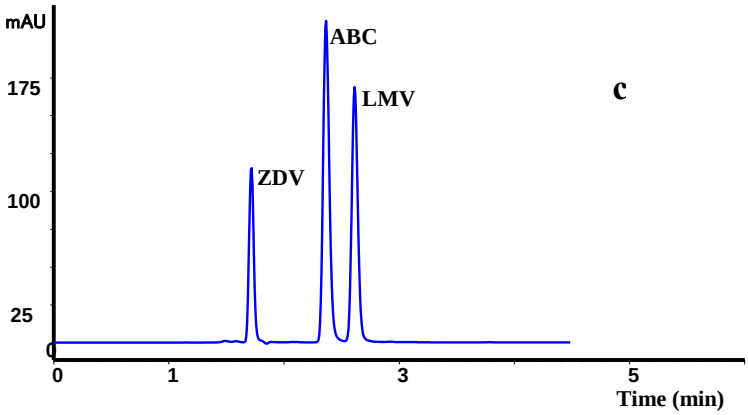
Supplementary material







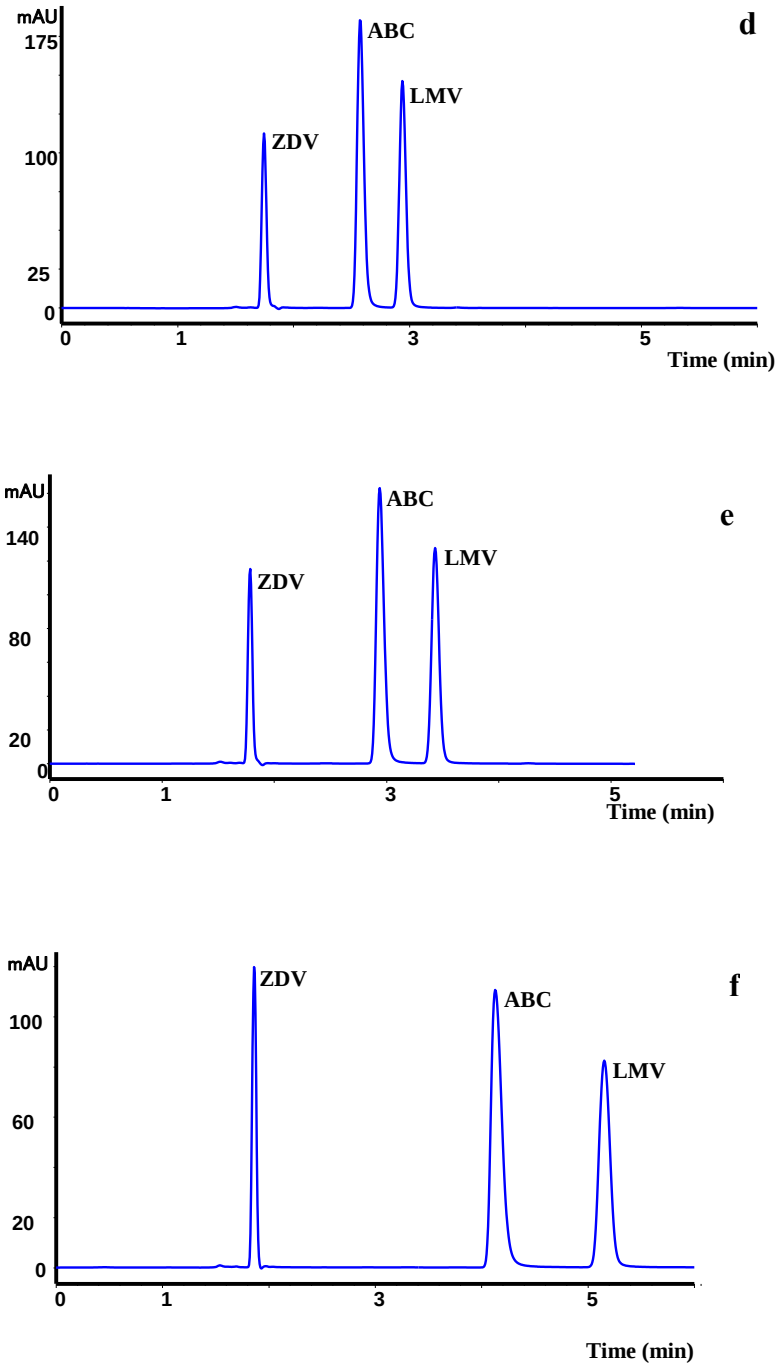
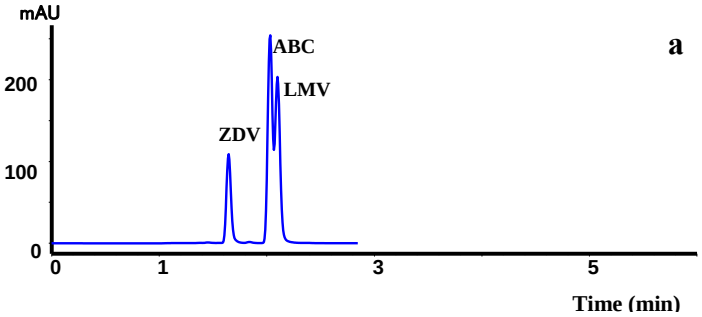


Figure S1. Kinetex HILIC (150 mm × 4.6 mm × 2.6 µm) column; 25 °C, flow rate: 1 mL min^{-1}, pH 6.68, wavelength: 280 nm, mobile phase: ACN:buffer solution- 20 mM ammonium acetate (v/v) (80:20) (a), (85:15) (b), (88:12) (c), (90:10) (d), (92:8) (e), and (95:5) (f).

Table S1. Comparison of the mobile phase compositions. Kinetex HILIC (150 mm × 4.6 mm, 2.6 µm) column; 25 °C, flow rate: 1 mL min^{-1}, and pH 6.6.

Percentage of ACN in the mobile phase	ZDV				ABC				LMV			
	t_R(min)	N	R_S	α	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α
80%	1.62	6757	-	-	1.98	10023	4,409	3.084	2.05	11038	1.266	1.192
85%	1.68	7392	-	-	2.17	9224	5.836	3.645	2.34	10983	1.881	1.248
88%	1.71	7891	-	-	2.36	9270	7.374	4.67	2.61	11621	2.56	1.292
90%	1.74	7999	-	-	2.57	8908	8.852	4.441	2.93	11884	3.358	1.34
92%	1.78	8595	-	-	2.93	8302	11.193	5.802	3.43	11832	3.873	1.353
95%	1.85	9872	-	-	4.12	7518	17.066	8.301	5.15	11178	5.343	1.397
98%	1.97	11998	-	-	14.29	10608	39.216	32.89	17.39	11280	4.98	1.236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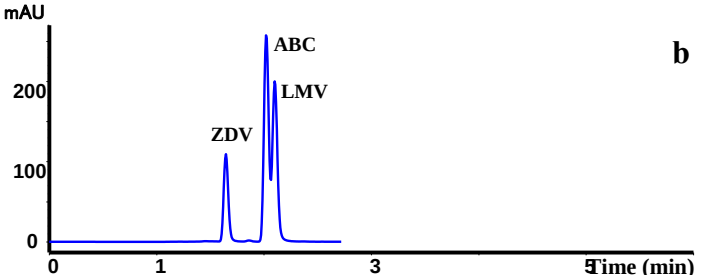
0
ABC
LMV
ZDV
a
0
1
3
5
Time (min)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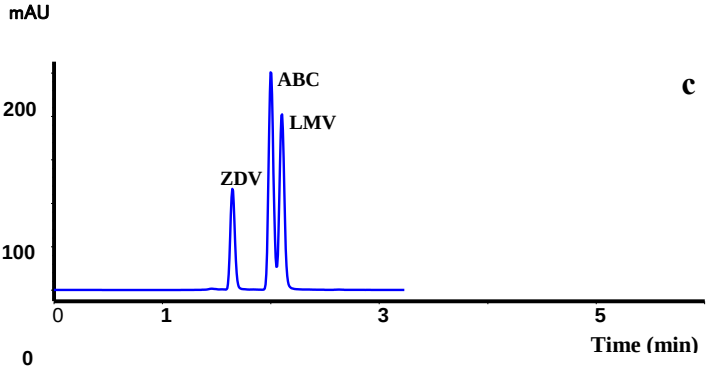
0
ABC
LMV
ZDV
b
0
1
3
5
Time (min)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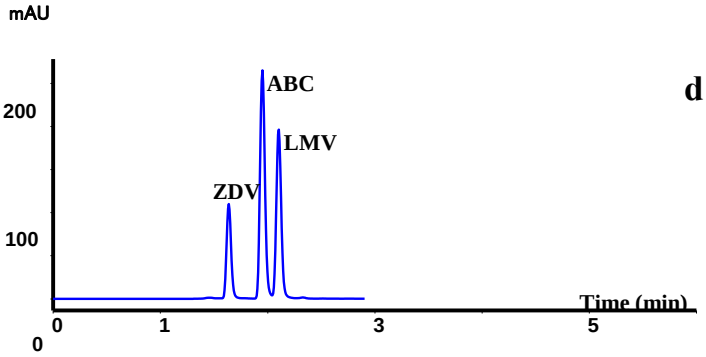
0
ABC
LMV
ZDV
c
0
1
3
5
Time (min)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ABC
LMV
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d
0
1
3
5
Time (min)

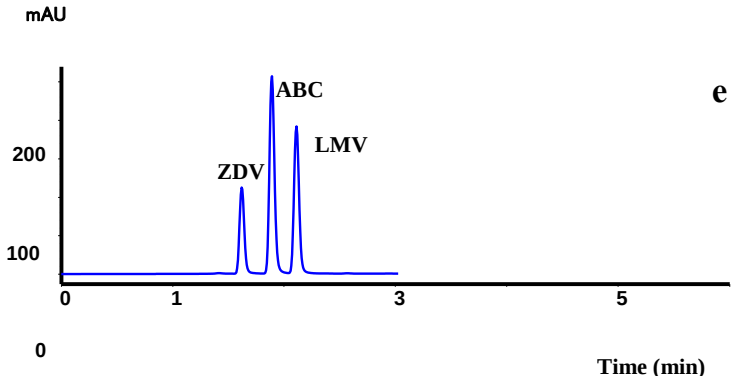


Figure S2. Kinetex HILIC (150 mm × 4.6 mm × 2.6 µm) column, 25 °C, flow rate: 1 mL min^{-1}, pH 6.68, wavelength: 280 nm, mobile phase: ACN:buffer solution- (5 mM) (a), (10 mM) (b), (20 mM) (c), (50 mM) (d), (100 mM), and (e) ammonium acetate (v/v). (80:20).

Table S2. Comparison of the concentration of the buffer solutions. Kinetex HILIC (150 mm × 4.6 mm × 2.6 μm) column, 25 °C, flow rate: 1 mL min^{-1}, pH 6.68, wavelength: 280 nm, and mobile phase: ammonium acetate (v/v) (80:20).

Buffer solution molarity	ZDV				ABC				LMV			
	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α
5 mM	1.64	6871	-	-	2.02	7352	3.09	4.42	2.09	11487	1.12	0.91
10 mM	1.64	6721	-	-	2.03	8771	4.49	3.22	2.10	10699	1.09	1.14
20 mM	1.62	6757	-	-	1.98	10023	4.40	3.084	2.05	11038	1.26	1.192
40 mM	1.63	6774	-	-	1.96	8535	4.01	2.92	2.09	9727	1.54	1.26
50 mM	1.63	6657	-	-	1.95	8710	3.81	2.85	2.10	10178	1.81	1.31
100 mM	1.65	6548	-	-	2.00	7959	3.26	2.46	2.10	9921	2.61	1.48

Table S3. Comparison of the pH of buffer solutions. Kinetex HILIC (150 mm × 4.6 mm × 2.6 µm) column; 25 °C, flow rate: 1 mL min^{-1}, pH 6.68, wavelength: 280 nm, and mobile phase: ammonium acetate (v/v) (80:20).

pH	ZDV				ABC				LMV			
	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α
5.50	1.63	6395	-	-	1.93	7558	3.61	2.63	2.12	9823	2.26	1.40
6.68	1.65	6548	-	-	2.00	7959	3.26	2.46	2.10	9921	2.61	1.48
7.78	1.62	6552	-	-	1.89	7524	3.04	2.42	2.13	9879	2.83	1.56

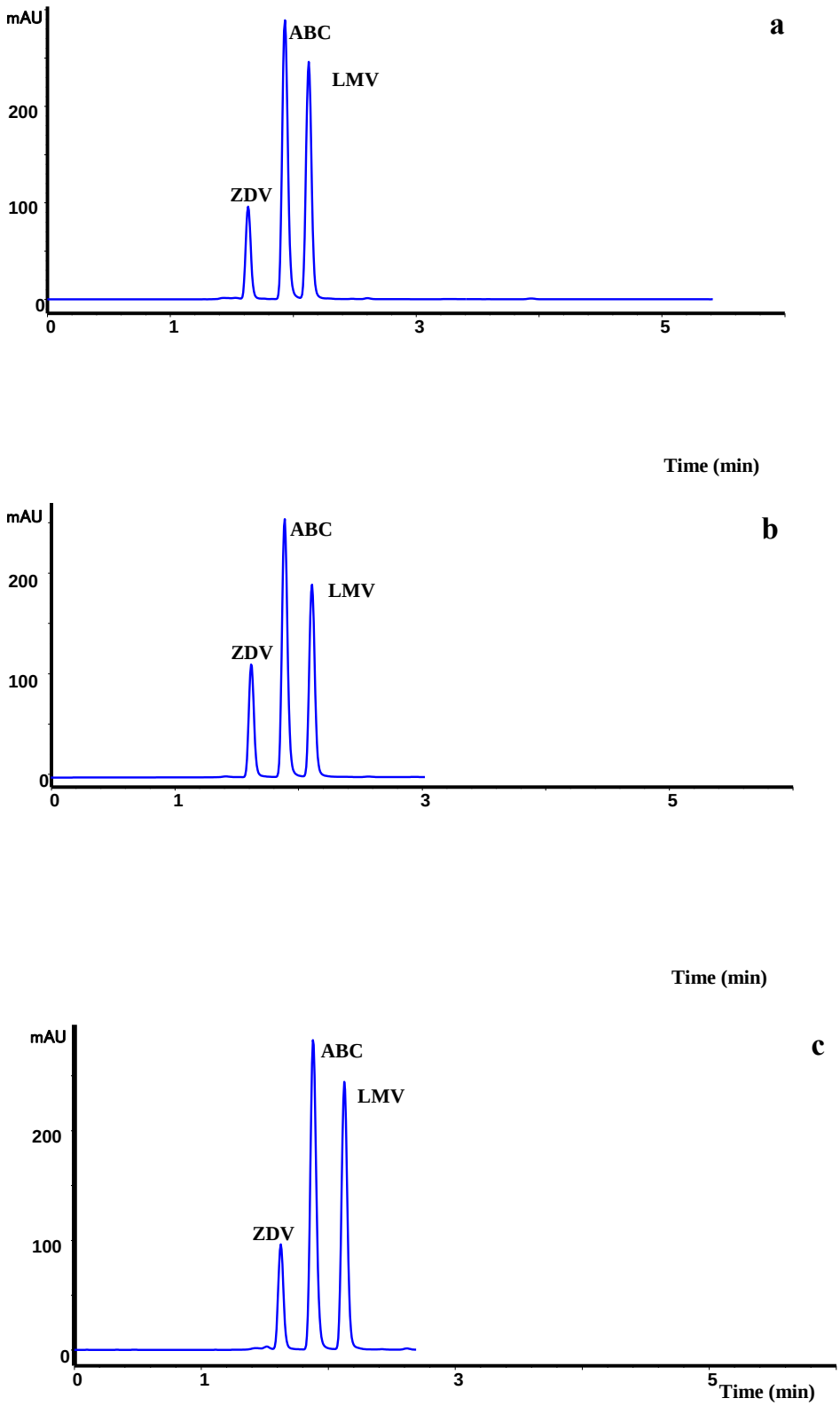


Figure S3. Kinetex HILIC (150 mm × 4.6 mm × 2.6 µm) column; flow rate: 1 mL min^{-1}, pH 5.50 (a), pH 6.68 (b), pH 7.8 (c), wavelength: 280 nm, and mobile phase: ACN:buffer solution- 100 mM ammonium acetate (v/v) (80:20).

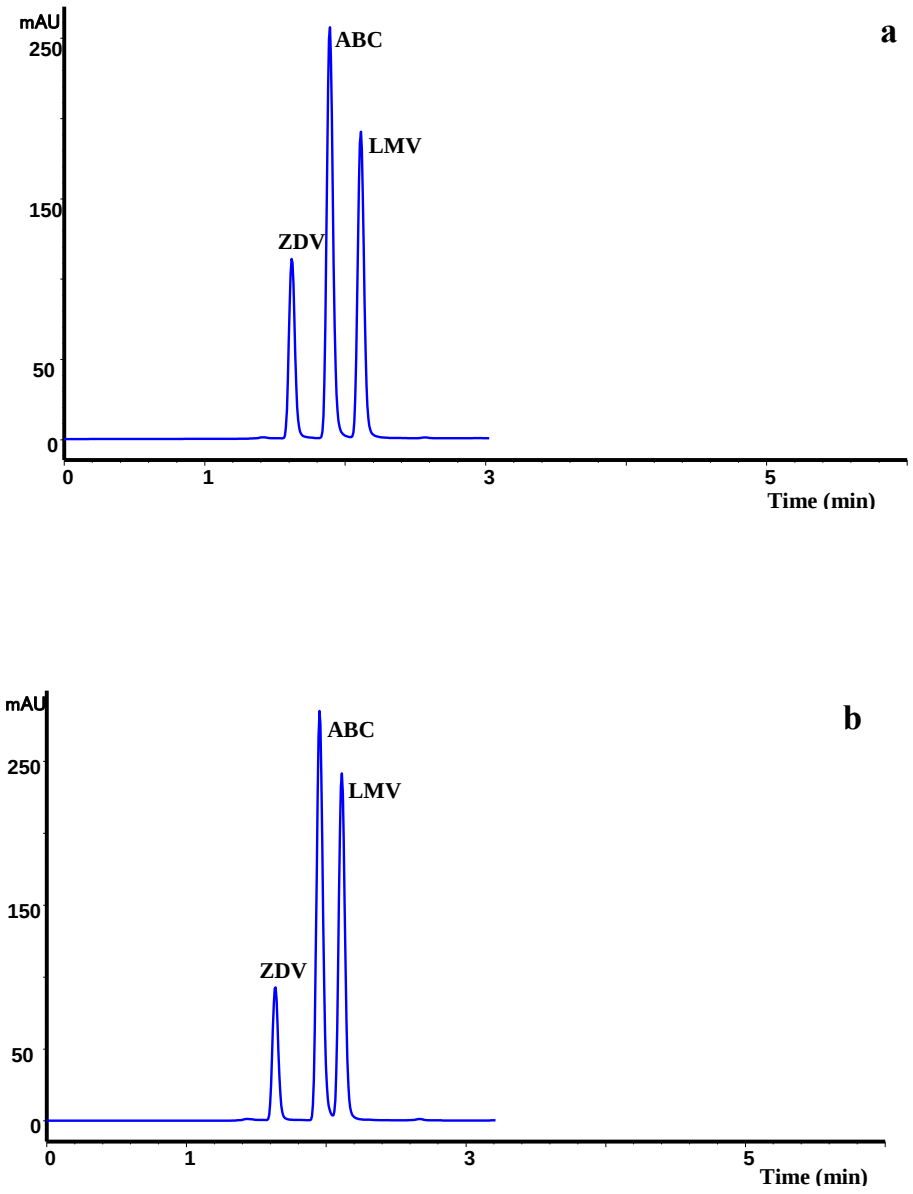


Figure S4. Kinetex HILIC (150 mm × 4.6 mm × 2.6 µm) column; flow rate: 1 mL min^{-1}, pH 6.68, wavelength: 280 nm, mobile phase: ACN:buffer- 100 mM ammonium acetate (a), 100 mM ammonium formate (b) (v/v) (80:20).

Table S4. Comparison of the buffer types. Kinetex HILIC (150 mm × 4.6 mm × 2.6 μm) column; flow rate: 1 mL min^{-1}, pH 6.68, wavelength: 280 nm, and mobile phase: ACN:buffer.

Buffer solution	ZDV				ABC				LMV			
	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α
Ammonium acetate	1.65	6548	-	-	2.00	7959	3.26	2.46	2.10	9921	2.61	1.48
Ammonium formate	1.65	6621	-	-	2.05	7789	3.44	1.42	2.09	9875	2.09	1.42

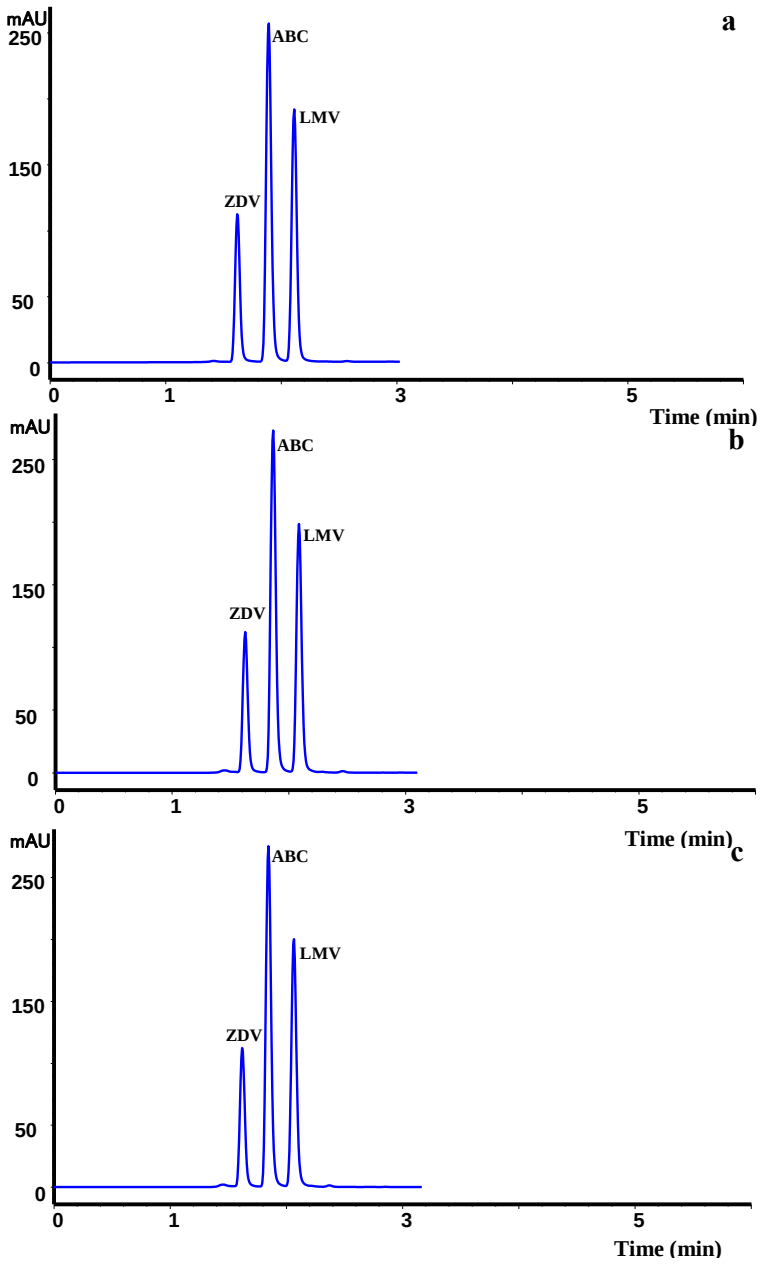


Figure S5. Kinetex HILIC (150 mm × 4.6 mm × 2.6 µm) column; 25 °C (a), 35 °C (b), and 45 °C (c); flow rate: 1 mL min^{-1}, pH 6.68, wavelength: 280 nm, and mobile phase: ACN:buffer solution-100 mM ammonium acetate (v/v) (80:20).

Table S5. Comparison of the temperatures. Kinetex HILIC (150 mm × 4.6 mm × 2.6 μm) column; flow rate: 1 mL min^{-1}, pH 6.68, wavelength: 280 nm, and mobile phase: ACN:buffer solution-100 mM ammonium acetate (v/v) (80:20).

Column temperature	ZDV				ABC				LMV			
	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α
25 °C	1.65	6548	-	-	2.00	7959	3.26	2.46	2.10	9921	2.61	1.48
35 °C	1.65	6501	-	-	2.01	7809	2.94	2.41	2.10	9978	2.63	1.54
45 °C	1.64	6398	-	-	2.01	7866	2.86	2.40	2.09	10075	2.63	1.52

Table S6. Comparison of the flow rates. Kinetex HILIC (150 mm × 4.6 mm, 2.6 µm) column; 25 °C, pH 6.68, and mobile phase: ACN:buffer solution-100 mM ammonium acetate (v/v) (80:20).

Flow rate (mL min^{-1})	ZDV				ABC				LMV			
	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α	t_R (min)	N	R_S	α
0.5	3.18	11659	-	-	3.73	10560	4.13	2.43	4.23	13957	3.47	1.54
0.75	2.13	8326	-	-	2.49	8363	3.55	2.37	2.82	10884	3.06	1.53
1	1.59	5977	-	-	1.87	6359	3.12	2.39	2.12	8489	2.63	1.51
1.25	1.28	4128	-	-	1.50	4814	2.85	2.37	1.70	6591	2.23	1.51
1.5	1.06	2873	-	-	1.24	3742	2.28	2.37	1.4	4537	1.98	1.51
2	0.79	2027	-	-	0.93	2380	1.83	2.39	1.05	3061	1.63	1.53

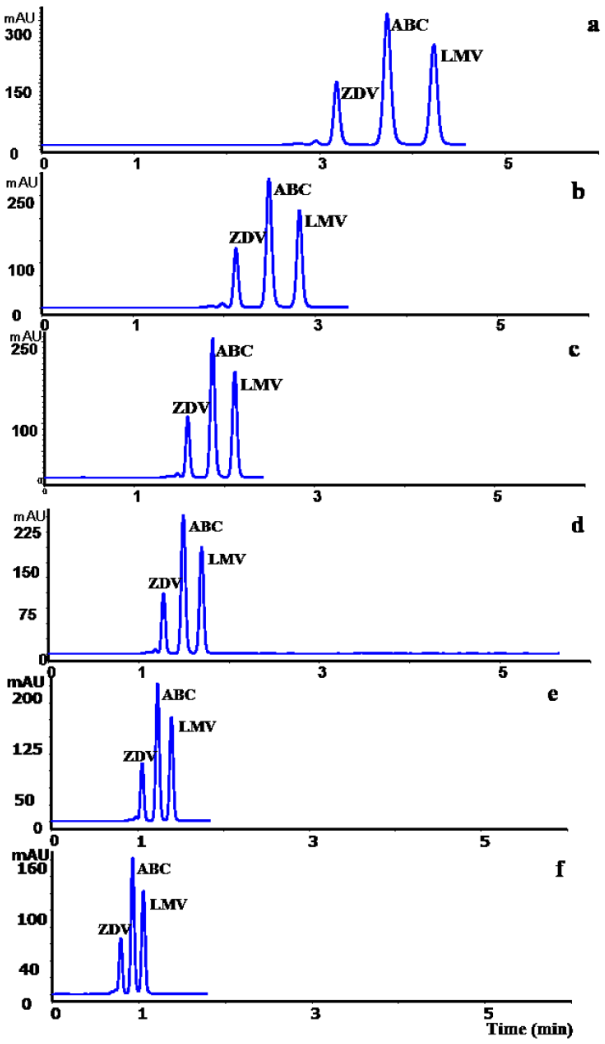
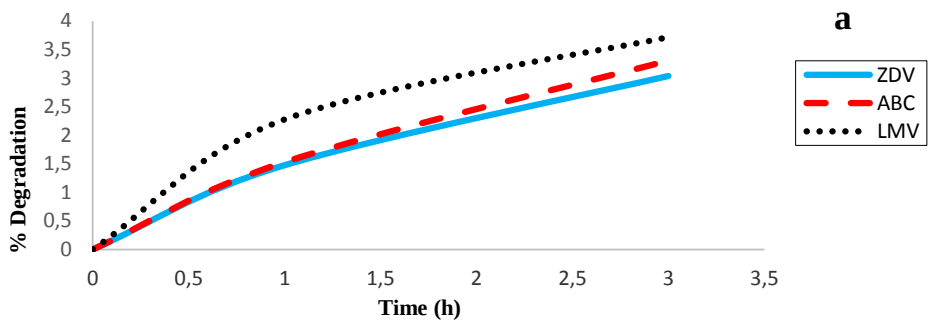
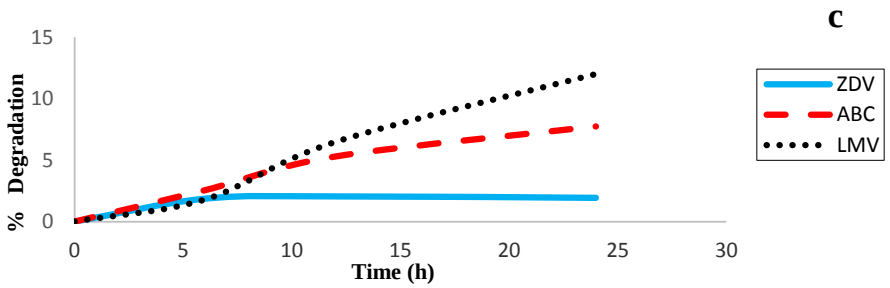


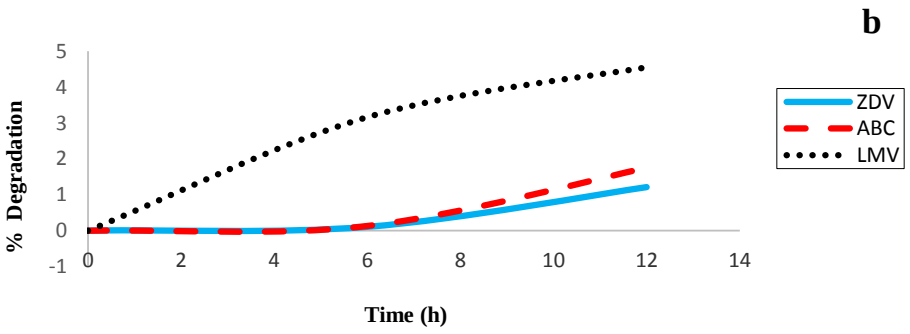
Figure S6. Kinetex HILIC (150 mm × 4.6 mm × 5 µm) column; flow rate: 0.5 mL min^{-1} (a), 0.75 mL min^{-1} (b), 1 mL min^{-1} (c), 1.25 mL min^{-1} (d), 1.5 mL min^{-1} (e), and 2 mL min^{-1} (f); pH 6.68; wavelength: 280 nm; and mobile phase: ACN:buffer solution-100 mM ammonium acetate (v/v) (80:20).

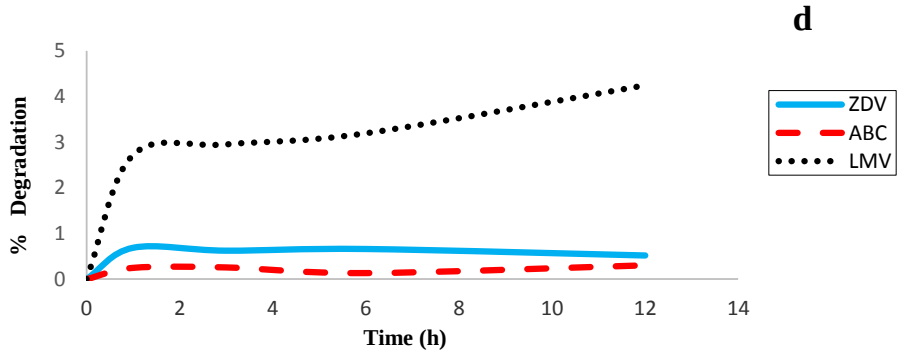
Table S7. System suitability test parameters under optimized conditions.

Parameters	ZDV	ABC	LMV
Retention time (min)	1.65	2.00	2.10
Capacity factor (k)	0.14	0.38	0.45
Resolution (R_s)	-	3.26	2.61
Theoretical plates (N)	6548	7959	9921
Selectivity factor (α)	-	2.46	1.48
Tailing factor	0.71	0.88	0.90
RSD% of retention time	0.12	0.17	0.21


a
% Degradation
Time (h)
ZDV
ABC
LMV


c
% Degradation
Time (h)
ZDV
ABC
LMV


b
% Degradation
Time (h)
ZDV
ABC
LMV


d
% Degradation
Time (h)
ZDV
ABC
LMV

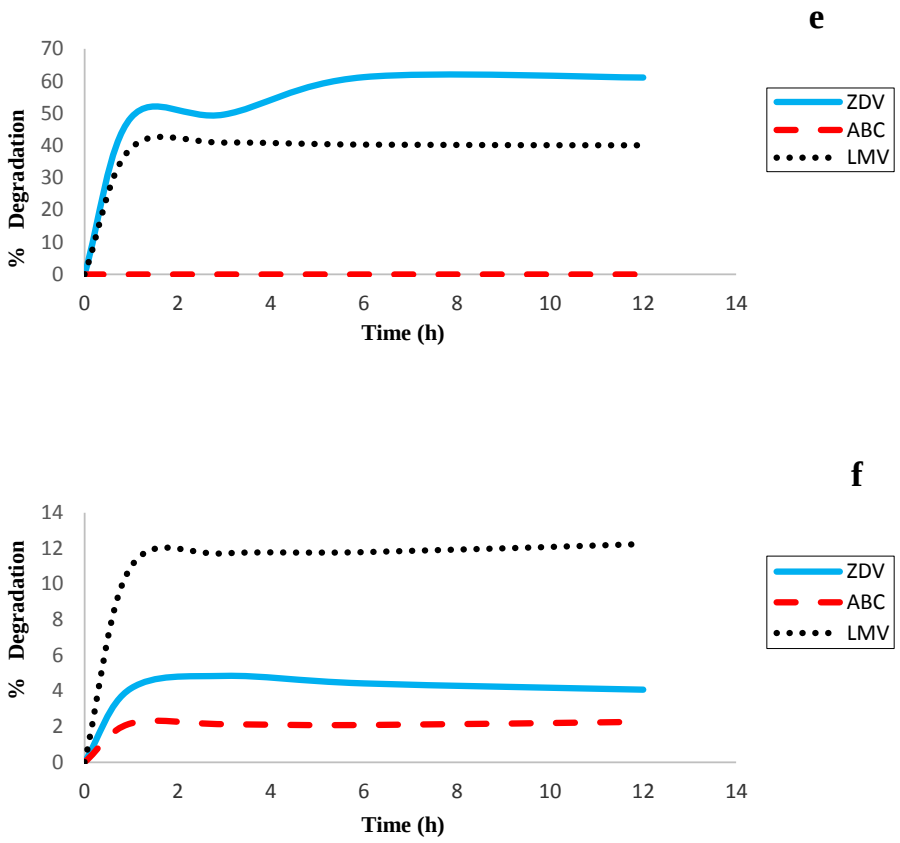


Figure S7. % degradation graphs of the active ingredients depending on the time of the run for the degradation: 80 °C hot water bath (a), UV light (254 nm) (b), 80 °C oven (c), acid hydrolysis with 0.1 M HCl (d), base hydrolysis with 0.1 M NaOH (e), and oxidation with 0.3% H_2O_2 (f).