RESEARCH ARTICLE

Determination of L-alanine isopropyl ester in tenofovir alafenamide fumarate by high performance liquid chromatography

Yanchuan Qiu, Lian Ma*

School of Pharmacy, Chongqing Medical and Pharmaceutical College, Chongqing, China.

Received: May 30, 2024; **accepted:** September 17, 2024.

L-alanine isopropyl ester is an inevitable specific impurity in the raw materials of the clinical drug remdesivir. This study aimed to develop a sensitive chromatographic method for the determination of L-alanine isopropyl ester content in remdesivir raw materials to ensure compliance with regulatory standards for this impurity level. The high-performance liquid chromatography analysis was performed on a Waters XSelect HSS T3 column (4.6 mm × 150 mm, 3.5 μm) with a mobile phase consisting of 20 mmol/L sodium heptane sulfonate solution containing 20 mmol/L potassium dibasic phosphate at pH 2.5 adjusted with phosphoric acid - acetonitrile (85:15) as mobile phase A and acetonitrile as mobile phase B. Gradient elution was employed at a flow rate of 1.0 mL/min with a detection wavelength set at 210 nm. The injection volume was 25 μL, and the column temperature was maintained at 40°C. The results showed that the separation between L-alanine isopropyl ester and adjacent peaks was excellent, and the linear range of the detection concentration was 2.10 - 63.00 μg/mL with a good linear relationship. The limits of quantification and detection were 2.10 μg/mL and 1.05 μg/mL, respectively. The average recovery rate was 100.7% and the relative standard deviation (RSD) was 5.0%. The test results for 3 batches of tenofovir alafenamide met the specification. This method is simple, sensitive, and reliable, and is well-suited for determining the content of L-alanine isopropyl ester in tenofovir alafenamide fumarate by HPLC analysis. It is applicable for the effective control of degradation impurities such as L-alanine isopropyl ester in the quality research process.

Keywords: tenofovir alafenamide; HPLC; L-alanine isopropyl ester.

***Corresponding author:** Lian Ma, School of Pharmacy, Chongqing Medical and Pharmaceutical College, Chongqing 401331, China. Email: maliany_1986@163.com.

Introduction

Tenofovir alafenamide fumarate (TAF) constitutes a novel nucleotide reverse transcriptase inhibitor, serving as a phosphoramidate prodrug of tenofovir [1]. It undergoes hydrolysis and phosphorylation within hepatocytes to exert its effect as tenofovir diphosphate. Additionally, TAF mitigates the deterioration of renal and bone density. Clinical research has manifested that patients availing TAF display stable renal function and bone density without marked deterioration. Comparative clinical studies have disclosed enhanced renal function, bone density, and more rapid recovery of hepatic function markers in patients transitioning from tenofovir dipifurate fumarate (TDF) to TAF, accentuating the superior safety and efficacy of the second-generation TAF over the first-generation TDF, rendering it more amenable for clinical application in hepatitis B patients [2, 3]. Furthermore, TAF can also be

"target" concentrated in the liver, reducing the drug concentration in the peripheral blood, which concurrently lowers the dose and frequency of TAF administration for patients, augmenting compliance.

Despite TAF being widely recognized as a clinical drug for managing chronic hepatitis B, its active pharmaceutical ingredient is highly susceptible to degradation during storage, giving rise to the formation of L-alanine isopropyl ester. Therefore, it is indispensable to determine the content of L-alanine isopropyl ester in the raw material of tenofovir alafenamide fumarate to ensure compliance with the relevant requirements in the Chinese Pharmacopoeia (2020 edition) and International Conference on Harmonization (ICH) guidelines on medicinal product registration [4, 5]. After literature searching, it has been ascertained that there are numerous reports on detection methods for related substances in tenofovir alafenamide fumarate [6-10]. However, reports on detection methods for L-alanine isopropyl ester in this drug's raw material are scarce. The literature had recourse to liquid chromatography-mass spectrometry (LC-MS) to determine the content of this specific impurity [6]. Although LC-MS is perceived as offering superior sensitivity for detection, it is cumbersome and costly. In contrast, high-performance liquid chromatography (HPLC) is easier to operate and involves more economically viable detection costs. Hence, establishing an HPLC method for determining the content of this specific impurity confers multiple advantages.

L-alanine isopropyl ester is an exceedingly polar compound that exhibits no retention in conventional reverse phase chromatography and cannot be discriminated from solvent peaks [10-12]. This study established and validated a simple and economically viable HPLC method through optimization of mobile phase composition, detector selection, diluent choice for the L-alanine isopropyl ester content determination in propofol tenofovir fumarate [13-18].

Materials and methods

Preparation of solutions

15% acetonitrile (Honeywell International Inc., Charlotte, North Carolina, USA) solution was used as diluent. The reference stock solution was prepared by dissolving 13 mg of L-alanine isopropyl ester hydrochloride (TRC, Co., Ltd., Toronto, Canada) into 100 mL of diluent. The reference solution was prepared by precisely transferring 1 mL of the reference stock solution into 9 mL of diluent and mixed well. The test solution was prepared by adding 100 mg of tenofovir alafenamide fumarate (Yaopharma Co., Ltd., Chongqing, China) sample into 10 mL of diluent.

Chromatographic conditions

Liquid chromatograph equipped with a highly sensitive ultraviolet detector (Shimadzu Corporation, Kyoto, Japan) was employed for this study. Waters XSelect HSS T3 chromatographic column with dimensions of 4.6 mm × 150 mm and a particle size of 3.5µm (Waters Corporation, Milford, MA, USA) was applied. The mobile phase A consisted of 20 mmol/L sodium heptane sulfonate solution containing 20 mmol/L potassium dihydrogen phosphate (pH 2.5, adjusted with phosphoric acid) - acetonitrile (85: 15), while the mobile phase B consisted of acetonitrile. Gradient elution was performed as A 100% for 0 - 15 min, A 100% - 25% for 15 - 16 min, A 25% for 16 - 22 min, A 25% - 100% for 22 - 23 min, A 100% for 23 - 30 min with the flow rate of 1.0 mL/min, detection wavelength of 210 nm, column temperature of 40°C, injection volume of 25 µL, and the injection disk temperature of 5°C.

Specificity tests

The spiked test solution was prepared with the concentration of L-alanine isopropyl ester equivalent to 100% of the reference solution. The tests were performed using HPLC with prepared 15% acetonitrile solution as blank, reference solution, and test solution.

Linear relationship inspection

1 mL of the reference solution was diluted with the diluent to generate linear solutions of various concentrations designated as #1 to #4 with # 5 was made by adding 1 mL of #3 to 9 mL diluent. The samples were measured by HPLC, and the peak areas were recorded. The linear regression was obtained by using the peak area as the ordinate (y) and the mass concentration (μg/mL) as the abscissa (x).

Quantification limit and detection limit test

The reference solution was diluted gradually and measured by HPLC until the signal-to-noise ratios of the L-alanine isopropyl ester peak reached 10:1 and 3:1, respectively. The quantification limit and detection limit were then obtained.

Precision, stability, repeatability tests

The precision test was done by measuring reference solution six consecutive times and recording the peak area of L-alanine isopropyl ester. The stability test was conducted by keeping reference solution and the test solution at 5℃ for 0, 6, 12, 24, and 36 h, respectively before measuring by HPLC. The proportion of the peak area of L-alanine isopropyl ester relative to that at 0 h was calculated. The repeatability test was performed by adding 100 mg of tenofovir alafenamide fumarate sample to 1 mL of the reference stock solution, diluting with 9 mL of diluent, and agitating thoroughly before loading on HPLC for measurement. A total of six samples were tested simultaneously.

Determination of recovery rate

100 mg of tenofovir alafenamide fumarate sample was dissolved in reference solution, while spiked test solutions of L-alanine isopropyl ester with concentrations of 50%, 100%, and 200% of the reference solution were also measured.

Durability test

The flow rate, column temperature, and pH value of the buffer in the mobile phase were adjusted to explore the influence of minor variations in chromatographic conditions. The 100% spiked test solution was employed to evaluate the resolution of L-alanine isopropyl ester from adjacent peaks in accordance with the modified chromatographic conditions. After obtaining the optimal conditions, three batches of tenofovir alafenamide fumarate raw material samples were used separately for method validation.

Results

Specificity, linear relationship, quantification and detection limits of proposed method

The results showed that the retention time of L-alanine isopropyl ester in the test solution was in line with that of the control sample. The adjacent peaks did not exert any hindrance on the determination. The blank solution did not bring any interference either (Figure 1), signifying that this method had remarkable specificity. The results demonstrated that the regression equation was as follows.

$$y = 0.0002X + 0.2001$$

The linear range extended from 2.10 to 63.00 μg/mL. The linear relationship was excellent with a r value of 1.0000 0. The results demonstrated that the quantification and detection limits of L-alanine isopropyl ester were 2.10 μg/mL and 1.05 μg/mL, respectively.

Precision, stability, and repeatability

The RSD of the peak area was 1.0% (n=6), signifying excellent instrument injection precision. When the reference solution was placed at 5℃ for 36 h, the peak area was within the range of 90% to 110% relative to 0 hour, indicating that the reference solution was stable after being placed at 5℃ for 36 h (Table 1). Six samples were concurrently prepared. The results showed that the RSD of L-alanine isopropyl ester was 1.5%, suggesting the favorable repeatability of the method (Table 2).

Recovery rate test

The results showed that the recovery rate of L-alanine isopropyl ester ranged from 90.0% to 105.0%, attaining an average recovery rate of 100.7% with an RSD of 5.0% (Table 3).

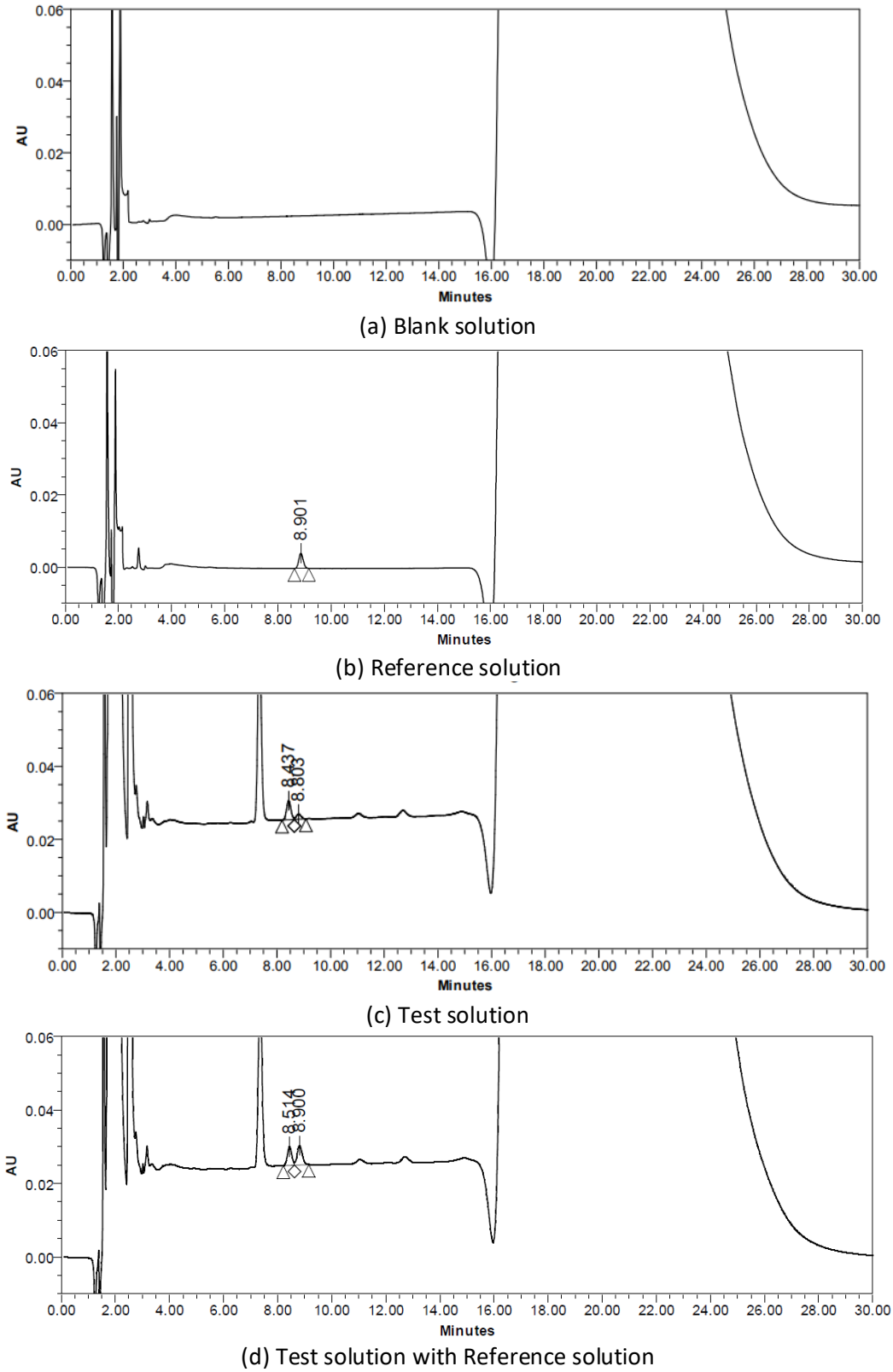

Figure 1. Typical HPLC chromatograms.

Table 1. Results of stability tests.

Sample	Time (h)	The area relative to 0 h (%)
The reference solution	0	-
	12	99.0
	36	97.5
The test solution	0	-
	6	106.0
	12	120.0

Table 2. Results of repeatability tests of L-alanine isopropyl ester.

Sample	Detected (%)	X (%)	RSD (%)
1	0.1348	0.1343	1.5
2	0.1369		
3	0.1335		
4	0.1316		
5	0.1328		
6	0.1359		

Table 3. Results of recovery rate tests of L-alanine isopropyl ester.

C (%)	Background (%)	Added (%)	Detected (%)	Recovery (%)	X (%)	RSD (%)
50	0.0264	0.0525	0.0736	90.0	100.7	5.0
		0.0526	0.0788	99.6		
100		0.0526	0.0808	103.4		
		0.1050	0.1348	103.2		
200		0.1052	0.1369	105.0		
		0.1052	0.1335	101.8		

Durability test

The results demonstrated that, under diverse chromatographic conditions, the degree of separation of L-alanine isopropyl ester from adjacent peaks exceeded 1.5. The detection was not influenced, indicating the good durability of proposed method (Table 4). The sample testing results showed that no L-alanine isopropyl ester was identified in all three batches of raw materials.

Discussion

Selection of mobile phase

L-alanine isopropyl ester is an extremely polar compound that remains unreserved in conventional reverse phase chromatography. The amino group in its structure exhibits weak alkalinity. Therefore, the mobile phase was adjusted to acidic conditions to ensure full protonation of the compound. By employing sodium heptane sulfonate as an ion pair reagent, the sulfonic acid adsorbed on the stationary phase bound to the amino group of L-alanine isopropyl ester, enabling its retention [19-22]. Considering the retention time, peak shape, and flushing time of L-alanine isopropyl ester, sodium heptanesulfonate/potassium dihydrogen phosphate solution (pH 2.5) - acetonitrile was selected as the initial ratio of the mobile phase,

Table 4. Results of durability tests.

Chromatographic conditions	Investigation	Separation
Velocity of flow (mL/min)	0.9	2.0
	1.1	1.7
Temperature (℃)	38	2.1
	42	1.8
pH	2.3	2.9
	2.7	2.2

followed by a gradient elution process. The initial proportion of the mobile phase was investigated in this study. The separation degree between the target peak and the adjacent peak was highly sensitive to the initial proportion.

Selection of detector and detection wavelength

L-alanine isopropyl ester has no strong UV absorption groups in its structure, mainly presenting UV terminal absorption. At the beginning of method development, different detectors were tested including ultraviolet absorption detector (UV) and evaporative light scattering detector (ELSD). After comparison, the sensitivity of the ELSD detector was relatively low compared to the UV detector, and its reproducibility was also poor. Therefore, in this study, a UV detector with a wavelength of 210 nm was chosen. To further enhance the detection sensitivity of the method, a high-sensitivity UV detector with a detection capacity 6 - 8 times higher than that of a regular UV detector was selected. When the ratio of sodium heptane sulfonate/potassium dihydrogen phosphate solution (pH 2.5) to acetonitrile was (83:17), the separation degree between the target peak and the adjacent peak was significantly inferior to that with the ration of 85: 15. Therefore, during the mobile phase preparation process, it was necessary to select a suitable measuring cylinder for mobile phase preparation to accurately control the initial proportion of the mobile phase.

Selection of diluent

Prophenol tenofovir fumarate is soluble in acetonitrile and methanol, slightly soluble in ethanol, and very slightly soluble in water. Therefore, in this study, acetonitrile that demonstrated relatively good dissolution of the bulk drug was chosen as one of the diluent components. The cut-off wavelength of acetonitrile was 190 nm, which was lower than that of other organic substances, and its impact on the baseline was relatively small. To minimize the solvent effects caused by significant differences in the proportion and flow of the organic phases in the diluent (solvent effects might lead to the deterioration or bifurcation of the peak shape of the substance to be measured), acetonitrile with a 15% diluent in the same proportion as the organic phase in the mobile phase was utilized to avoid solvent effects.

Selection of column temperature

In the early stage of method development, the separation degree between the target peak and adjacent peaks was examined under column temperatures of 45℃, 40℃, 35℃, and 30℃, respectively. The results demonstrated that the higher the temperature, the better the column efficiency of the target peak and the greater the degree of separation from the adjacent peaks. Finally, 40℃ was selected as the column temperature for this method.

The method established in this study for the determination of L-alanine isopropyl ester in the raw material of propofol tenofovir fumarate was simple, sensitive, reliable, and cost-effective, which held certain advantages over the methods reported in the past. This method could be employed to control the degradation of impurity

L-alanine isopropyl ester during the quality determination of the raw material of tenofovir alafenamide fumarate.

References

1. Aloy B, Tazi I, Bagnis CI, Gauthier M, Janus N, Launay-Vacher V, *et al*. 2016. Is tenofovir alafenamide safer than tenofovir disoproxil fumarate for the kidneys? Aids Rev. 18(4):184-192.
2. Sampath R, Zeuli J, Rizza S, Temesgen Z. 2016. Tenofovir alafenamide fumarate for the treatment of HIV infection. Drug Today. 52(11):617-625.
3. Chon HY, Ahn SH, Kim YJ, Yoon JH, Lee JH, Sinn DH, *et al*. 2021. Efficacy of entecavir, tenofovir disoproxil fumarate, and tenofovir alafenamide in treatment-naive hepatitis B patients. Hepatol Int. 15(6):1328-1336.
4. National Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China. Volume 4. Edited by Chinese Pharmacopoeia Commission. Beijing: China Medical Science and Technology Press. 2020:483-485.
5. International Technical Requirements for Drug Registration. Quality Part. Edited by Zhou HJ. Beijing: People's Health Publishing House. 2000:82-89.
6. Woldemariam G, Kyad A, Moore S, Qiu J, Semin D, Tan ZJ, *et al*. 2020. Development and validation of a HPLC-UV method for urea and related impurities. PDA J Pharm Sci Technol. 74(1):2-14.
7. Wolfender JL. 2009. HPLC in natural product analysis: the detection issue. Planta Med. 75(7):719-734.
8. Mochizuki R, Higashi K, Okamoto Y, Abe H, Iwase H, Toida T. 2019. Detection of selenocyanate in biological samples by HPLC with fluorescence detection using könig reaction. Chem Pharm Bull (Tokyo). 67(8):884-887.
9. Soyseven M, Arli G. 2022. Method validation and rapid determination of monosodium glutamate in various food products by HPLC-Fluorescence detection and method optimization of HPLC-Evaporative light scattering detection approach without derivatization. J Chromatogr Sci. 60(8):760-769.
10. Wang MD, Hou DY, Lv GT, Li RX, Hu XJ, Wang ZJ, *et al*. 2021. Targeted *in situ* self-assembly augments peptide drug conjugate cell-entry efficiency. Biomaterials. 278:121139.
11. Sun C, Wang Z, Yang K, Yue L, Cheng Q, Ma YL, *et al*. 2021. Polyamine-responsive morphological transformation of a supramolecular peptide for specific drug accumulation and retention in cancer cells. Small. 17(43):2101139.
12. Dong Y, Tu Y, Wang K, Xu C, Yuan Y, Wang J. 2020. A general strategy for macrotheranostic prodrug activation: synergy between the acidic tumor microenvironment and bioorthogonal chemistry. Angew Chem Int Edit. 59(18):7168-7172.
13. Xu S, Luo S, Zhu Z, Xu J. 2019. Small molecules as inhibitors of PCSK9: Current status and future challenges. Eur J Med Chem. 162:212-233.
14. Ma X, Bi E, Lu Y, Su P, Huang C, Liu L, *et al*. 2019. Cholesterol induces CD8+ T cell exhaustion in the tumor microenvironment. Cell Metab. 30(1):143-156. e5.
15. Lei Y, Dai W, Liu Z, Guo S, Cai Z, Shi J, *et al*. 2018. A novel strategy for realizing dual state fluorescence and low-temperature phosphorescence. Mater Chem Front. 3(2):284-291.
16. Di Pilato M, Kim EY, Cadilha BL, Prüßmann JN, Nasrallah MN, Seruggia D, *et al*. 2019. Targeting the CBM complex causes Treg cells to prime tumors for immune checkpoint therapy. Nature. 570(7759):112-116.
17. Zhou J, Du X, Chen X, Wang J, Zhou N, Wu D, *et al*. 2018. Enzymatic self-assembly confers exceptionally strong synergism with NF-κB targeting for selective necroptosis of cancer cells. J Am Chem Soc. 140(6):2301-2308.
18. Yang J, Zhai S, Qin H, Yan H, Xing D, X Hu. 2018. NIR-controlled morphology transformation and pulsatile drug delivery based on multifunctional phototheranostic nanoparticles for photoacoustic imaging-guided photothermal-chemotherapy. Biomaterials. 176:1-12.
19. Row RD, Prescher JA. 2018. Constructing new bioorthogonal reagents and reactions. Acc Chem Res. 51(5):1073-1081.
20. Li B, Lu L, Zhao M, Lei Z, Zhang F. 2018. An efficient 1064 nm nir-ii excitation fluorescent molecular dye for deep-tissue high-resolution dynamic bioimaging. Angewandte Chemie. 57(25):7483-7487.
21. Zhang Y, Ultsch M, Skelton NJ, Burdick DJ, Beresini MH, Li W, *et al*. 2017. Discovery of a cryptic peptide-binding site on PCSK9 and design of antagonists. Nat Struct Mol Biol. 24(10):848-856.
22. Eder T, Stangl T, Gmelch M, Remmerssen K, Laux D, Höge S, *et al*. 2017. Switching between H- and J-type electronic coupling in single conjugated polymer aggregates. Nat Commun. 8(1):1641.