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RP-HPLC Method for Simultaneously Quantifying the Antiviral Drug Contents of Acyclovir, Amantadine, and Oseltamivir in Pharmaceutical Formulations

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Abstract

Antibiotics treat bacterial infections, whereas antiviral drugs treat viral illnesses. Antivirals are used to treat a variety of infectious diseases, including COVID-19 and influenza. The technique of reversed-phase high-performance liquid chromatography (RP-HPLC) provides more sensitivity and precision than other approaches, particularly spectrophotometric. This research seeks to develop a simple method for simultaneously quantifying acyclovir, amantadine, and oseltamivir in creams and capsules. The RP-HPLC method optimization and development for verifying the separation and quantification of three antiviral drugs included the investigation of the optimal buffer concentration, pH value, and acetonitrile content. On a C8 HyperClone BDS column, the RP-HPLC system with UV detection achieved separation (250 x 4.60 mm, 130A, and 5). The mixture of acetonitrile and acetate buffer as mobile phase gradient elution at a detection wavelength of 254 nm and 1 mL/min flow rate. The proposed method provided a linear range of 0.08-10.5, 0.06-4.5 and 0.02-14 µg/mL, as well as excellent validated values for LOD (0.0205, 0.0107, and 0.0083 µg/mL) and LOQ (0.0621, 0.0324 and 0.0251 µg/mL) with a coefficient of determination (r2) of the regression line of 0.9998, 0.9988, and 0.9994 for acyclovir, amantadine, and oseltamivir, respectively.

Keywords: RP-HPLC, antiviral drugs, oseltamivir, acyclovir, amantadine.

طريقة RR–HPLC للقياس الكمي المتزامن لمحتويات الأدوية المضادة للفيروسات للأسيكلوفير والأمانتادين والأوسيلتاميفير في المستحضرات الصيدلانية

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الخلاصة

تعالج المضادات الحيوية الالتهابات البكتيرية، بينما تعالج الأدوية المضادة للفيروسات الأمراض الفيروسية. تستخدم مضادات الفيروسات لعلاج مجموعة متنوعة من الأمراض المعدية ، بما في ذلك COVID–19 والإنفلونزا. توفر تقنية كروماتوغرافيا السائل عالية الأداء ذات المرحلة العكسية (RP–HPLC) حساسية ودقة أكثر من الطرق الأخرى ، وخاصة قياس الطيف الضوئي. يسعى هذا البحث إلى تطوير طريقة بسيطة للقياس المتزامن للأسيكلوفير والأمانتادين والأوسيلتاميفير في الكريمات والكبسولات. تضمن البحث تحسين وتطوير

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طريقة RP–HPLC للتحقق من الفصل والتقدير الكمي لثلاثة عقاقير مضادة للفيروسات التحقيق في تركيز المخزن المؤقت الأمثل وقيمة الأس الهيدروجيني ومحتوى الأسيتونيتريل. في عمود C8 HyperClone BDS ، حقق نظام RP–HPLC المزود بالكشف عن الأشعة فوق البنفسجية فصلًا (250 × 4.60 مم و 130 أمبير و 5). خليط الأسيتونيتريل وخلات العازلة كتدرج طور متحرك شطف بطول موجة كشف يبلغ 254 نانومتر ومعدل تدفق 1 مل / دقيقة. قدمت الطريقة المقترحة نطاقًا خطيًا من 0.08–10.5 و 0.06–4.5 و 0.02–14 ميكروغرام / مل ، بالإضافة إلى قيم تم التحقق من صحتها ممتازة لـ 0.0205) LOD و 0.0107 و 0.0083 ميكروغرام / مل) و 0.0621) LOQ و 0.0324 و 0.0251 ميكروغرام) / مل) مع معامل تحديد (r2)لخط الانحدار 0.9998 و 0.9988 و 0.9994 للأسيكلوفير والأمانتادين والأوسيلتاميفير ، على التوالي.

1. Introduction

The increasing prevalence of antiviral drug resistance is a significant public health concern. Due to the tendency for strains to become more virulent, suitable therapeutic procedures are necessary [1,2]. Several antiviral medications have been discovered, and in addition to treating individuals, farmers now use them to protect their flocks [3,4]. Concerns emerged, however, regarding the increasing prevalence of antiviral drug resistance [5-7]. One of the hypothesised causes is the inappropriate use of antiviral drugs in human treatment during avian influenza and severe acute respiratory syndrome (SARS) outbreaks [8,9]. In addition, it has been established that oseltamivir carboxylate is neither removed nor degraded during regular sewage water treatment and is released into the environment [10,11]. This study chose three antiviral medications: acyclovir, amantadine, and oseltamivir (Figure 1). Acyclovir is a synthetic antiviral analogue of 2-deoxyguanosine. It is an effective inhibitor of herpes simplex virus types 1 and 2 and varicella-zoster virus, which remain frequent viral diseases in humans. In the past decade, the incidence and severity of infections caused by Herpes simplex virus (HSV) have grown due to an increase in immunocompromised individuals due to severe chemotherapy regimens, increased organ donation, and increased human immunodeficiency virus infections [12-15].

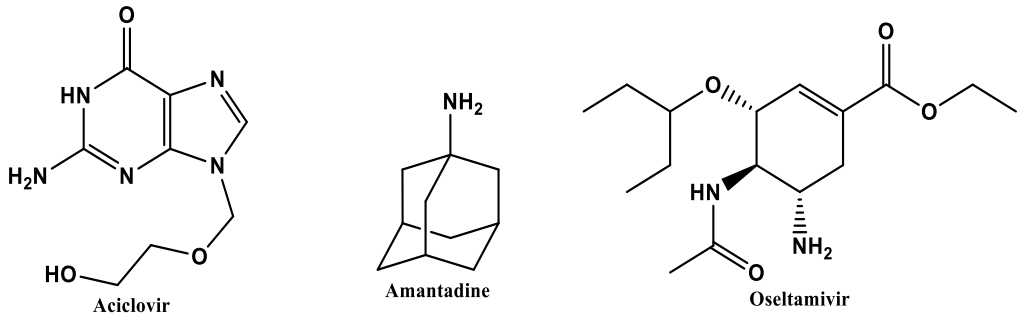

Figure 1: Chemical structures of acyclovir, amantadine, and oseltamivir

The antiviral drug amantadine has a tricyclic aliphatic ring structure with an amino group as its principal functional component. Clinically, it is used to treat influenza A, Parkinson's disease, hepatitis C, multiple sclerosis, and drug-induced extrapyramidal reactions [16]. The central nervous system's specific method of action is not understood. Evidence suggests that amantadine improves dopamine release and absorption equilibrium by blocking the *N*-methyl-D-aspartate receptor [17]. This helps to reduce the symptoms of Parkinsonism [18] and multiple sclerosis [19]. Due to its antiviral properties for treating influenza, amantadine has been widely used in the chicken farming sector [20]. As an antiviral medicine, oseltamivir treats all influenza viruses. It is the most effective antiviral medication for all influenza viruses that may infect humans, including pandemic strains. It has also been utilised in Korea as a therapeutic against SARS-CoV-2 and MERS-CoV [21,22]. It has been shown that early administration of

oseltamivir in combination with antibiotic therapy lowers the duration of fever and the interval between the fever's peak and decline in outpatients with suspected COVID-19 [23,24]. Various chromatographic methods for quantifying acyclovir, amantadine, and oseltamivir in multiple applications have been reported. Using UPLC BEH Amide and Agilent SB-Aq columns, ultra-high-performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) [25,26] was utilized to determine acyclovir, amantadine, and oseltamivir in chicken tissues and muscle. Multiple methods have been developed for detecting antiviral medicines in bodily fluids [27,28]. Using Diamonsil-5 m and Novaflex C18 columns and HPLC with fluorescence and ultraviolet detection, antiviral drugs in human plasma were determined [29-31] using fluorescence and ultraviolet detection. Several HPLC methods for quantifying acyclovir, amantadine, and oseltamivir in pharmaceutical formulations have been published. Determining the concentrations of acyclovir, amantadine, and oseltamivir using a time- and cost-efficient HPLC technique with UV detection was thus our aim. Avoiding lengthy sample preparation steps and substituting acetonitrile and acetate buffer for methanol in the mobile phase composition in a pharmaceutical analytical laboratory would save costs while maintaining appropriate sensitivity for routine analysis.

2. Materials and methods

2.1. Materials and chemicals

All of the chemicals used were of analytical grade. Standard compounds of acyclovir, amantadine, and oseltamivir were obtained from Sigma-Aldrich (Darmstadt, Germany). Acetic acid, sodium acetate, and acetonitrile were obtained from Carl Roth (Karlsruhe, Germany). Using a Milli-Q system, water with a resistivity of at least 18.2 Mcm was created (Millipore, Billerica, MA, USA).

2.2. Preparation of standard solutions

Individual stock standards (50 µg/mL) were prepared by dissolving 5 mg of acyclovir, amantadine, and oseltamivir in 100 mL of the corresponding mobile phase (acetonitrile and buffer acetate). The stock solution was diluted to the necessary concentration with the mobile phase to prepare working standard solutions. From the stock solutions of acyclovir, amantadine, and oseltamivir, a working solution (17 µg/mL) was made up of millipore water in a brown flask. The calibration standards for acyclovir, amantadine, and oseltamivir used were in the range of 0.02 to 14 µg/mL.

2.3. HPLC instrumentation and conditions

A Merck-Hitachi (Germany) system with an L-6200 pump and an L-4200 UV/Vis detector was used. Data analyses were done using the N2000 Photographic Data Workstation Module Integrator. The chromatographic separation was performed at 25 °C on a C8 HyperClone BDS reversed-phase column (250 × 4.60 mm, 130A, and 5µ). The flow rate of the mobile phase was 1 mL/min and comprised a combination of acetonitrile and a 0.01 M acetate buffer (pH = 4.75). In gradient elution, the mobile phase composition gradually increases the acetonitrile content from 5 to 50% (v/v) with a constant concentration of the buffer at 0.01 M at pH 4.75. For detecting acyclovir, amantadine, and oseltamivir, a wavelength of 254 nm was used. The volume of the injection was 10 µL.

2.4. Sample preparation

Commercially available samples for acyclovir, amantadine, and oseltamivir are as follows: For acyclovir, we obtained three samples (15 g) of the creams from different companies: VIRADERM® (Sama Alfayhaa, Iraq), Avir (Brawn Laboratories, India), and AcicloCareTM (AdvaCare, USA). Each gram of cream contained 50 mg of acyclovir. Two grams of an

accurately weighed sample containing 100 mg of acyclovir were dissolved in 50 mL of 0.01 M aqueous NaOH and transferred to a 100 mL volumetric flask, and 500 µg/mL was the final concentration of acyclovir. The samples were treated with ultrasonication and 40°C heat for forty minutes. From the resultant solution, 0.5 mL was diluted into 50 ml of the mobile phase. Then, a concentration of acyclovir equal to 5 µg/mL was extracted. For amantadine, the three capsule samples came from different companies: Amantrel-Cipla-India, Symmetrel- Novartis-Switzerland and Amantadine.HCl-Morningside Pharmaceuticals Ltd. (UK) were obtained (each capsule contained 100 mg of amantadine). As for oseltamivir, the three capsule samples were obtained from different companies: Flumivir®-Benta sal-Lebanon, Antiflu-Cipla-India, and ENFLUVIR-ATABY-Turkey, (each capsule contained 75 mg oseltamivir). Each capsule's net weight was determined after weighing fifteen capsules. The powder from 100 and 75 mg of amantadine and oseltamivir capsules was weighed precisely, dissolved in the mobile phase, and transferred to a 250 mL volumetric flask. In an ultrasonic bath, the mixture was sonicated for 10 minutes; 400 and 300 µg/mL were the final concentrations of amantadine and oseltamivir, respectively. From the resultant solution, 0.5 mL was diluted into 50 mL of the mobile phase. Then, a concentration of amantadine and oseltamivir equal to 4 and 3 µg/mL was administered. The solutions were filtered through a membrane filter (0.45 µm).

2.5. Method validation

The validation process was carried out according to ICH guidelines [32] and the United States Pharmacopeia [33]. The parameters used to validate the method of analysis were: linearity, limits of detection (LOD), limits of quantification (LOQ), accuracy, precision, F-test, and t-test.

3. Results and discussion

3.1. Method development

The HPLC method linked with UV detection has been developed to provide a suitable solution for the routine quality control analysis of this multicomponent antiviral medication mixture. The most challenging aspect of HPLC method development is achieving satisfactory analyte resolution with well-defined symmetrical peaks in a reasonable amount of time. To reach this purpose, several variations of acetonitrile content, eluent, and pH buffer concentration were made to optimize the mobile phase.

3.1.1. The influence of acetonitrile fraction on antiviral drugs retention

Mobile phase compositions were changed systematically by variation of the acetonitrile content from 5% to 50% (v/v) with a constant concentration of the buffer at 0.01 M at pH 4.75 (Figure 2). The antiviral drugs acyclovir, amantadine, and oseltamivir showed an increasing rate of retention with increasing aqueous phase (acetate buffer) [34]. Increasing the polarity of the mobile phase by increasing the proportion of the aqueous phase (buffer) enhances the hydrophilic interaction between the stationary phase and solutes, hence facilitating solute elution. The hydrophilicity of the antiviral drugs is responsible for hydrophilic interactions [35]. The values of the antiviral drugs are evident from the logPow. This is explained in logPow acyclovir, amantadine, and oseltamivir values, respectively (-1.04, -1.5, and -1.84) [36].

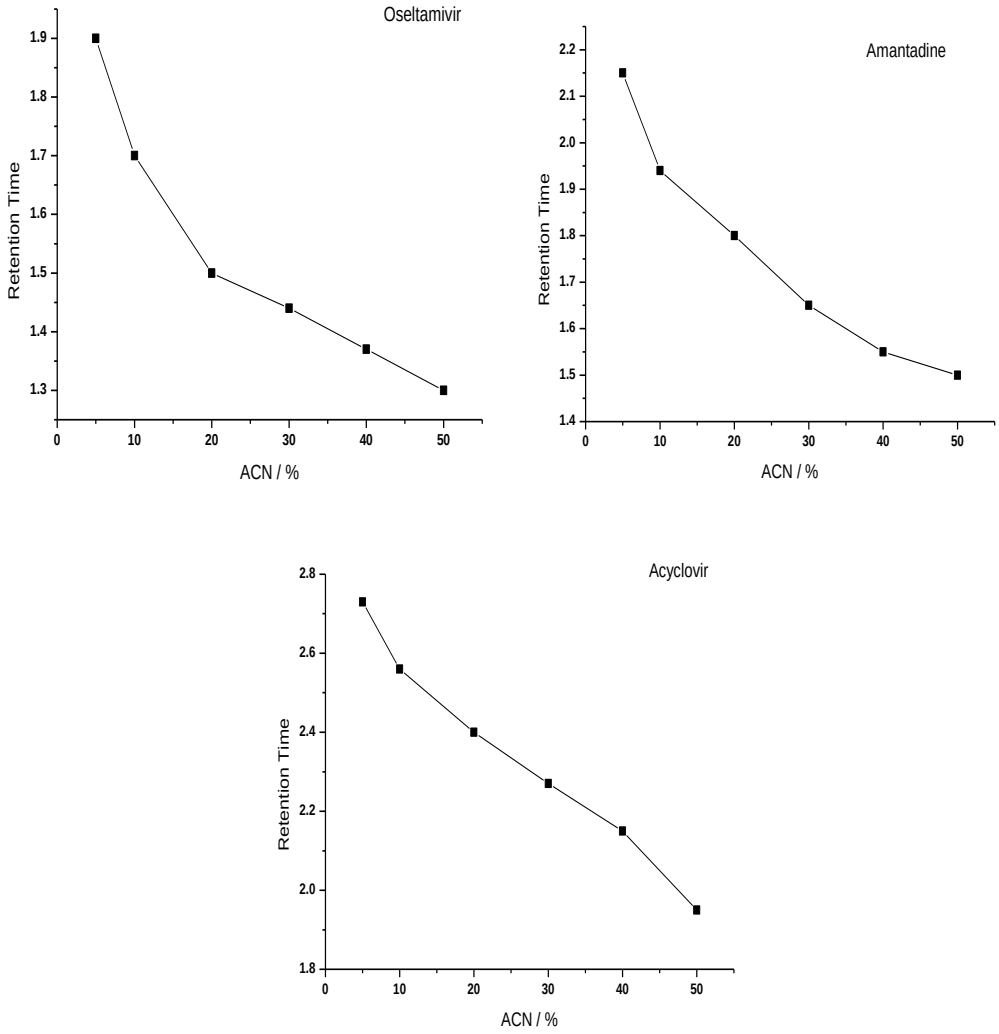

Figure 2: Effect of the acetonitrile fraction used in the mobile phase on the determination of acyclovir, amantadine, and oseltamivir.

3.1.2. The influence of acetate buffer pH on antiviral drugs retention

The reality that the ionisation state of antiviral medicines changes over the pH range of the aqueous phase (acetate buffer) can present practical obstacles. The pH of the mobile phase can also play a significant role in determining retention and selectivity. The retention behavior of the antiviral drugs under RP-HPLC conditions is depicted in Figure 3. The buffer pH was changed between 3.5 and 5.5 with a constant buffer concentration of 0.01 M and an acetonitrile concentration of 5%. The retention time of antiviral drugs decreased as the pH buffer increased from 3.5 to 5.5. The functional group (amine group) of antiviral medications is protonated in a mobile phase with a pH of 4.75 that lies below the isoelectric points of acyclovir, amantadine, and oseltamivir (between 7.5 and 11.65) and antiviral medicines, as are cationic forms [36, 37].

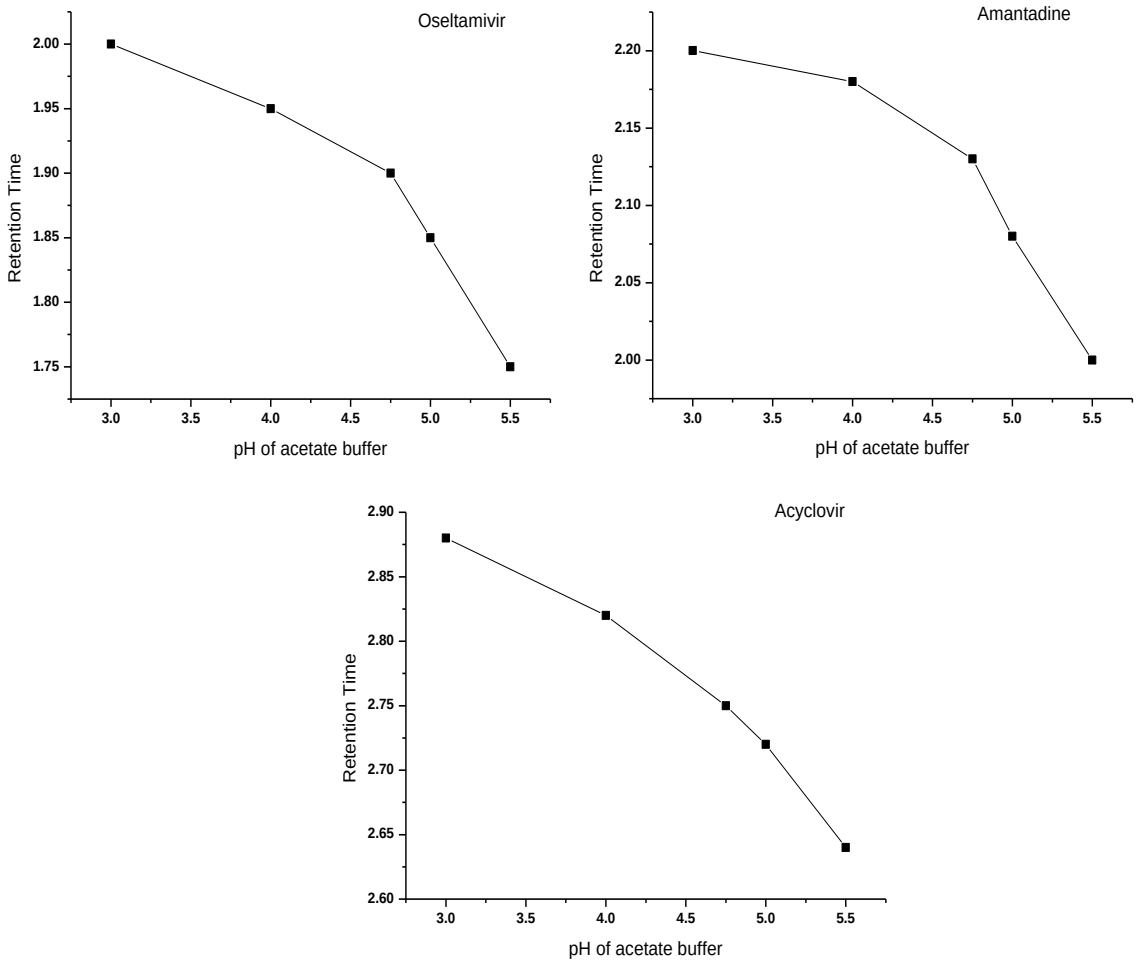

Figure 3: Effect of the acetate buffer pH used in the mobile phase on the determination of acyclovir, amantadine, and oseltamivir.

3.1.3. The influence of acetate buffer concentration on antiviral drugs retention

Polar molecules are eluted more rapidly when buffer concentration increases. This can be used to separate co-eluting peaks, although greater concentrations increase column viscosity, resulting in high back pressure [35,38]. At the conclusion of the optimization circumstances, the buffer concentration was altered from 0.01 to 0.025 M at pH 4.75, while the acetonitrile concentration remained constant at 5%. Figure 4 demonstrates the decreased retention time of antiviral drugs with increased buffer concentration. A rise in buffer concentration diminishes the retention of positively charged antiviral drugs.

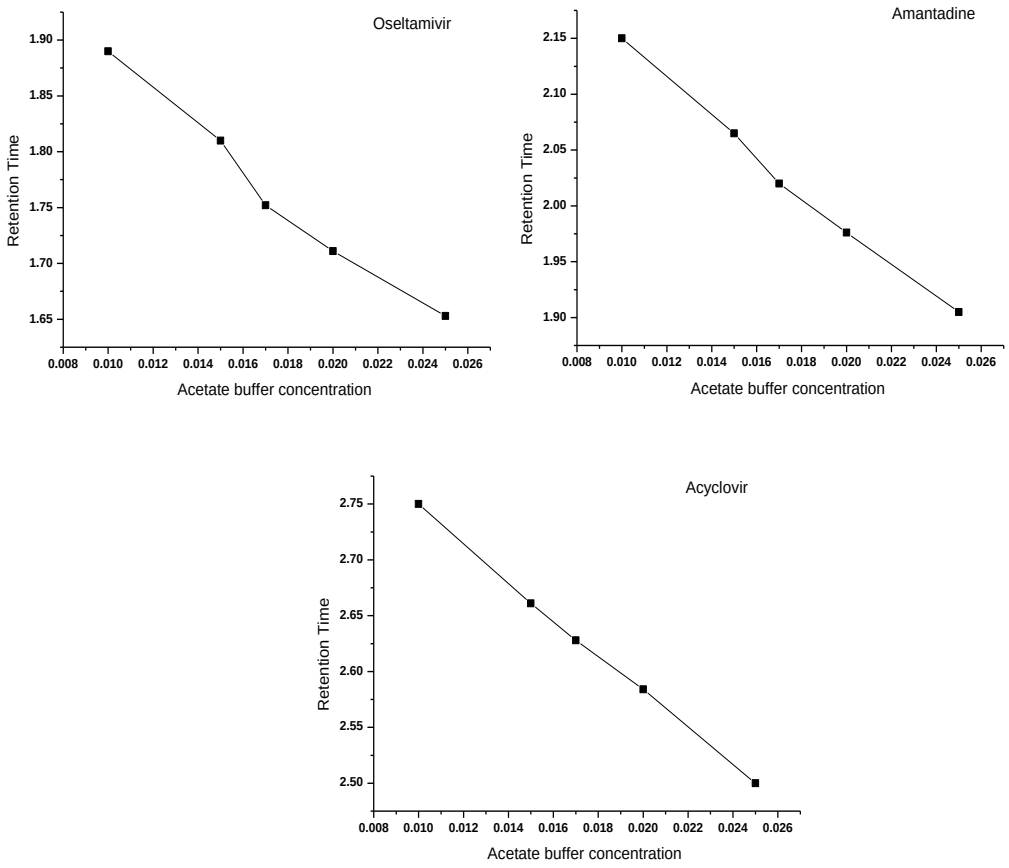

Figure 4: Effect of the acetate buffer concentration used in the mobile phase on the determination of acyclovir, amantadine, and oseltamivir.

Figure 5 depicts the chromatogram of the separation of acyclovir, amantadine, and oseltamivir at 5% acetonitrile and 95% 0.01 M acetate buffer (pH = 4.75) after determining the optimal conditions.

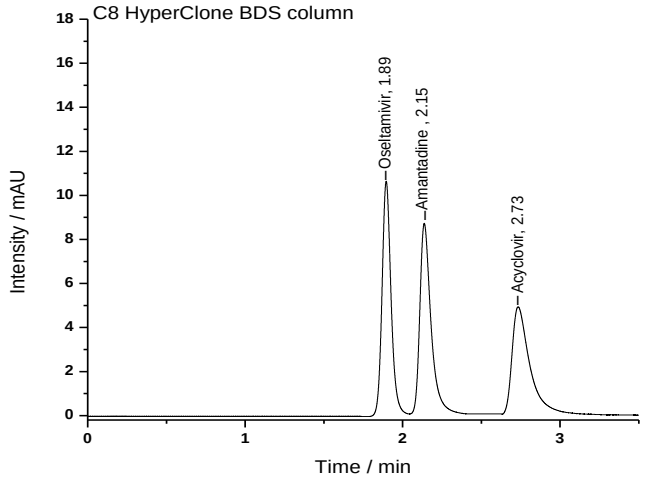

Figure 5: Chromatogram for the separations of 0.1 µg/mL (acyclovir, amantadine, and oseltamivir)

3.2. Calibration graph

Acyclovir, amantadine, and oseltamivir calibration graphs have been developed in optimal conditions (eluent: 0.01 M acetate buffer at pH 4.75 and 5% acetonitrile) by plotting peak area versus acyclovir, amantadine, and oseltamivir concentrations and displaying the 0.08-10.5, 0.06-4.5, and 0.02-14 µg/mL range concentration, respectively (Figure 6).

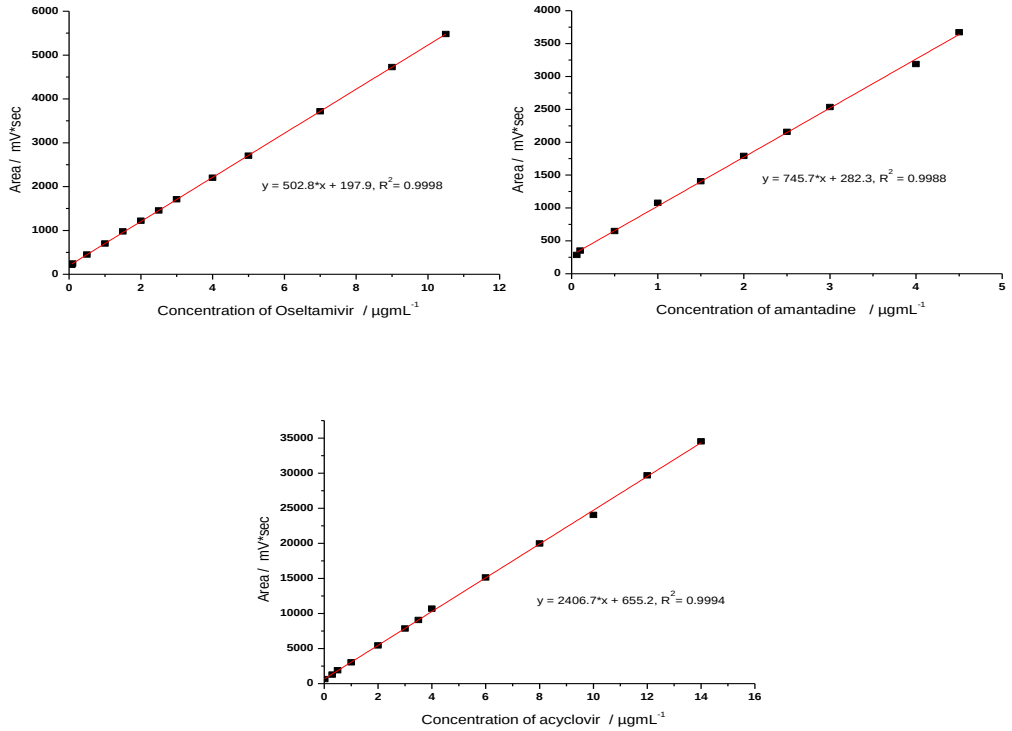

Figure 6 : Calibration curves for the determination of acyclovir, amantadine, and oseltamivir.

3.3. Statistical information analysis

Table 1 contains the direct calibration graphs and the statistical results for determining acyclovir, amantadine, and oseltamivir in RP-mode. The proposed method has verified the procedure for three concentrations throughout the range. Each concentration was repeated three times, and five consecutive days' worth of calibration samples was assessed. The precision and accuracy of RSD and recovery values were determined in intra-day and inter-day investigations (Table 2).

Table 1: Analytical values of statistical treatments for determination of acyclovir, amantadine, and oseltamivir from the calibration graph

Parameter	Oseltamivir	Amantadine	Acyclovir
Linearity Range (µg/mL)	0.08-10.5	0.06-4.5	0.02-14
Regression Equation	y=502.8*x+197.9	y=745.7*x+282.3	y=2406.7*x+655.2
Determination Coefficient (r2)	0.9998	0.9988	0.9994
LOD* (µg/mL)	0.0205	0.0107	0.0083
LOQ (µg/mL)**	0.0621	0.0324	0.0251

**Limit of detection (LOD = 3.3 σ/S, where σ is the standard deviation of the intercept and S is the slope of the calibration lines); **Limit of quantitation (LOQ = 10 σ/S)*

Table 2: The accuracy and precision of the proposed method for the determination of acyclovir, amantadine, and oseltamivir

	Intra-day n = 5			Inter-day n = 25		
Taken (µg/mL)	**Found (µg/mL)**	**Rec. (%)**	**RSD (%)**	**Found (µg/mL)**	**Rec. (%)**	**RSD (%)**
			Acyclovir			
0.700	0.698	99.71	0.51	0.698	99.71	0.50
0.900	0.894	99.33	0.40	0.893	99.22	0.44
1.300	1.305	100.38	0.32	1.308	100.61	0.37
			Amantadine			
0.700	0.703	100.42	0.38	0.703	100.42	0.45
0.900	0.895	99.44	0.33	0.896	99.55	0.40
1.300	1.295	99.61	0.22	1.297	99.76	0.35
			Oseltamivir			
0.700	0.690	98.57	0.42	0.692	98.85	0.48
0.900	0.906	100.66	0.40	0.905	100.55	0.41
1.300	1.297	99.76	0.38	1.296	99.69	0.45

3.4. Determination of antiviral drugs in marketed formulation

The findings of the acyclovir, amantadine, and oseltamivir determinations using three pharmaceutical preparations (capsules and creams) containing the target medications at the indicated concentrations of 2 and 500 mg, respectively, are shown in Table 3 (Figure 7). The data acquired utilizing the RP mode (Table 3) were compared to the United States Pharmacopeia protocol [33] using the student t-test and variance F-test with a confidence level of 95%. The estimated t and F values (Table 4) did not surpass the theoretical values, showing that the method's accuracy and precision for determining acyclovir, amantadine, and oseltamivir in dosage forms were not markedly different.

Table 3: Validation of the proposed method for determining the concentrations of acyclovir, amantadine, and oseltamivir in pharmaceutical products

Name of pharmaceutical	Manufacturer	Present conc. (µg/mL)	Found (µg/mL)	Rec. (%)	RSD (%) n=5
Acyclovir					
VIRADERM®-cream	Sama Alfayhaa-Iraq	5	4.95	99.00	1.23
Avir-cream	Brawn Laboratories Ltd-India	5	5.02	100.40	0.92
AcicloCare™ cream	AdvaCare-USA	5	4.97	99.40	0.85
Amantadine					
Amantrel-capsule	Cipla-India	4	3.98	99.50	0.33
Symmetrel-capsule	Novartis-Switzerland	4	4.03	100.75	0.42
Amantadine.HCl-capsule	Morningside Pharmaceuticals Ltd-UK	4	4.07	101.75	0.30
Oseltamivir					
Flumivir®-capsule	Benta sal-Lebanon	3	2.93	97.66	0.97
Antiflu-capsule	Cipla-India	3	2.98	99.33	0.77
ENFLUVIR-capsule	ATABY-Turkey	3	3.06	102.00	0.52

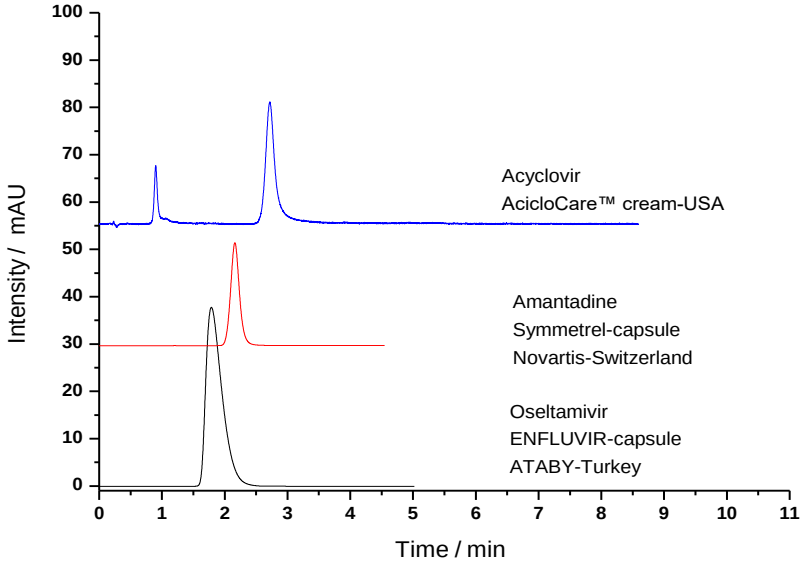

Figure 7: Chromatogram for the separations of antiviral drugs in marketed formulations

Table 4: A comparative study of the proposed RP-HPLC technique with standard methods for quantifying acyclovir, amantadine, and oseltamivir using the t-test and F-test

Name of pharmaceutical	Suggested method RP-HPLC	Standard methods [33]	t-Test* (tab.)**	F-Test* (tab.)**
Acyclovir				
VIRADERM®-cream	99.00	99.78	0.4527 (2.7764)**	0.8124 (19.0000)**
Avir-cream	100.40	99.54		
AcicloCare™ cream	99.40	101.03		
Amantadine				
Amantrel-capsule	99.50	99.35	0.7630 (2.7764)**	1.2880 (19.0000)**
Symmetrel-capsule	100.75	101.33		
Amantadine.HCl-capsule	101.75	100.48		
Oseltamivir				
Flumivir®-capsule	97.66	98.56	0.7923 (2.7764)**	2.69045 (19.0000)**
Antiflu-capsule	99.33	101.06		
ENFLUVIR-capsule	102.00	100.62		

**Calculated, **tabulated values*

4. Conclusion

This article outlines a straightforward, sensitive, specific, and generic RP-HPLC-UV method for quantifying three antiviral medications simultaneously. The medications under investigation are acyclovir, amantadine, and oseltamivir. The statistical output of the studies provided outstanding linearity, precision, accuracy, and specificity. As mentioned earlier, the acceptable analytical performance of the suggested approach strengthens its appropriateness for routine drug analysis in quality control laboratories. Consequently, the proposed RP-HPLC methodology can be utilised for routine analysis of antiviral medicines in medicinal dosages.

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