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Ultra-performance liquid chromatography method for quantitative analysis of nystatin and triamcinolone acetonide in topical creams after *in vitro* release using franz diffusion cell

Seelam Jayadev[1], Ismail Yusuff[1*], Faiyaz Shakeel[2] and Ubaidulla Uthumansha[1]

Abstract The accurate quantification of active ingredients in topical creams is critical for ensuring efficacy, safety, and quality. Therefore, this initiative is to develop and validate a robust ultra-performance liquid chromatography (UPLC) method for the quantification of nystatin (Nys) and triamcinolone acetonide (TA) in topical creams. Validation of the in vitro release test (IVRT) apparatus and UPLC method was conducted according to standard requirements. IVRT apparatus demonstrated exceptional control over key parameters, aligning with stringent standards, thus ensuring consistent and reproducible drug release profiles. Membrane inertness evaluation confirmed no significant binding of Nys and TA. The proposed UPLC method was found to be linear in the range of 0.65–31.93 µg/mL for TA and 17.67-863.27 IU/mL for Nys with determination coefficients of 1.0000 for both drugs, enabling accurate measurement across a wide range of drug concentrations. Recovery rates and mass balance results were within acceptable ranges, validating the method's accuracy. The IVRT method exhibited low day-1 and day-2 variability, underscoring its reliability. Sensitivity and specificity were comparable to similar studies, demonstrating the method's applicability in distinguishing between different formulation strengths and variations. The method's robustness was confirmed by its resistance to variations in dose amount, receptor media composition, stirring speed (stirring speed is controlled by rotation speed controller connected to the vertical diffusion cell Instrument. Material of construction is plastic, plastic bead is connected to the helix spring and placed in the cell for uniform mixing.), and temperature. The UPLC method validation affirmed its high sensitivity and reliability for detecting low levels of active ingredients, with excellent selectivity, specificity, linearity, precision, accuracy, stability, and robustness. The IVRT equipment's and UPLC analytical method's thorough certification and validation procedures verify its fit for the precise and dependable measurement of Nys and TA in topical cream compositions. These confirmed techniques satisfy all scientific and legal criteria.

Clinical trial number Not Applicable.

Keywords IVRT method, Nystatin, Triamcinolone acetonide, UPLC, topical cream

*Correspondence:
Ismail Yusuff
ismailcsp@crescent.education
[1]Crescent School of Pharmacy, B.S. Abdur Rahman Crescent Institute of Science and Technology, G.S.T Road, Vandalur, Chennai 600048, Tamil Nadu, India
[2]Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia

Introduction

Topical creams are widely used in dermatology due to their direct application to the skin, providing localized treatment with minimal systemic absorption. They are essential in managing a variety of skin conditions, offering therapeutic benefits such as anti-inflammatory, antifungal, and antibacterial effects [1]. Among the numerous active ingredients used in topical formulations, nystatin (Nys) and triamcinolone acetonide (TA) stand out for their significant roles in treating dermatological conditions [2]. Nys, an antifungal agent, and TA, a corticosteroid, are often combined in topical creams to harness their complementary therapeutic effects [3, 4]. Nys is primarily used to treat fungal infections, while TA is effective in reducing inflammation and allergic reactions [3]. The combination of these two agents in a single formulation aims to provide comprehensive treatment for inflammatory dermatoses complicated by fungal infections [4]. Nys is a polyene antifungal antibiotic derived from *Streptomyces noursei*. It works by binding to ergosterol, a crucial component of fungal cell membranes, thereby disrupting membrane integrity and causing cell death [5]. Nys is highly effective against *Candida* species, making it a popular choice for treating cutaneous, mucocutaneous, and systemic fungal infections [6]. Topical creams containing Nys are commonly prescribed for conditions such as cutaneous candidiasis, diaper dermatitis, and intertrigo [7]. These creams help alleviate symptoms by eradicating the fungal pathogens responsible for the infection, thus promoting healing and comfort for the patient [8]. TA is a synthetic corticosteroid with potent anti-inflammatory and immunosuppressive properties. It also inhibits the release of proinflammatory cytokines and mediators, reducing inflammation, redness, and itching associated with various skin conditions. Its effectiveness makes it a cornerstone treatment for a range of dermatological disorders [9]. This corticosteroid is frequently used in topical formulations to manage conditions such as eczema, psoriasis, dermatitis, and allergic reactions [9].

The rationale for combining Nys and TA in a single topical formulation lies in their complementary therapeutic actions [10]. Inflammatory dermatoses are often complicated by secondary fungal infections, necessitating a treatment that can address both inflammation and fungal overgrowth simultaneously [11]. This combination offers several benefits, including simplified treatment regimens, improved patient adherence, and enhanced therapeutic outcomes. By targeting both the inflammatory and infectious components of a condition, the combined formulation can provide faster relief and more comprehensive management of symptoms [12].

Accurate quantification of active ingredients in pharmaceutical formulations is crucial to ensure efficacy, safety and quality. Analytical method plays a vital role in pharmaceutical industry by providing reliable data on the concentration of active ingredients, helping to maintain consistent therapeutic effects [13]. Developing analytical methods for combined topical creams presents unique challenges due to the presence of multiple active ingredients and excipients [14]. Ensuring specificity, sensitivity, and accuracy in the quantification process is essential to meet regulatory standards and ensure patient safety [15, 16].

Literature survey revealed a spectrometry method for the determination of TA in its tablet and injectable dosage form [17]. A spectrometry method has also been reported for the determination of Nys in its pharmaceutical preparations [18]. Some spectrometry methods have been reported for the simultaneous determination of Nys and TA in their synthetic mixtures and commercial products [19–21]. Various high-performance liquid chromatography (HPLC) methods have also been reported to determine TA in its pharmaceutical products [11, 22–25]. A HPLC method has also been reported to quantify Nys in its pharmaceutical products [26]. Some HPLC methods have also been reported to for the simultaneous determination of Nys and TA in their synthetic mixture and topical creams [12, 27]. A HPLC method has also been reported to for the simultaneous determination of Nys and TA in industrial wastewater [28]. A high-performance thin-layer chromatographic approach has also been reported to determine TA in the presence of its impurities and degradation products [24]. An ultra-performance liquid chromatography (UPLC) method has also been reported to determine TA in the presence of its degradation products [29]. However, the validation of UPLC methods have not been reported to determine Nys and TA in topical creams after in vitro release using Franz diffusion cell. As a result, the aim of this work is to create and validate a UPLC method for TA and Nys quantification in topical creams after in vitro release using Franz diffusion cell. This approach seeks to produce accurate, dependable, repeatable findings meeting regulatory criteria and so guarantee the efficacy and quality of the combined product. The effective development and validation of this analytical technique will help to improve quality control procedures in the pharmaceutical sector.

IVRM procedure involves the Franz vertical diffusion cells and various in vitro release testing (IVRT) have been employed for different pharmaceutical formulations. Acyclovir cream (5%) was tested using a vertical diffusion cell with a polysulfone membrane and a 0.9% NaCl solution as the receptor medium [30]. Similarly, Zovirax cream was evaluated using a USP type-II immersion cell, incorporating a polysulfone membrane and an alkaline borate buffer (pH 9.2) as the receptor medium [31]. Acyclovir ointment was analyzed with a USP type-II

immersion cell utilizing a nylon membrane and a pH 7.4 phosphate-buffered saline solution [32]. For betamethasone dipropionate ointment, a Franz diffusion cell was used with a polysulfone membrane and a receptor medium composed of ethanol, isopropyl alcohol (IPA), acetonitrile, and hexane [33]. Acyclovir cream formulation was assessed using a vertical diffusion cell with various membranes, including nylon, tuffryn, durapore, and nitrocellulose, and a normal saline (0.9% w/v NaCl) receptor medium [34]. Metronidazole cream was tested using a vertical diffusion cell with a tuffryn membrane and a 0.9% NaCl receptor medium [35]. Nitroglycerine patches were evaluated using the USP paddle method without a membrane, employing DE aerated water as the receptor medium [36]. Hydrocortisone acetate cream underwent testing in a vertical diffusion cell with a tuffryn membrane and an ethanol-water receptor medium [37]. Cyclosporine ophthalmic ointment was studied using Franz diffusion cells with a polyether sulfone membrane and a receptor medium consisting of pH 7.4 phosphate-buffered saline (PBS), 0.5% sodium dodecyl sulfate (SDS), and 20% ethanol [38].

Materials and methods

Materials

Nys and TA were procured from E-Merck (Mumbai, India). Tetrahydrofuran, acetonitrile, and methanol for UPLC were procured from Local vendor in Chennai (Tamil Nadu, India). Water of UPLC grade was obtained from the Milli-Q system, which was used in the preparation of the buffer and sample solutions. The marketed Nys and TA cream USP (Taro) was procured from the local pharmacy in Chennai (Tamil Nadu, India). The formulation contains TA at a concentration of 1 mg per gram and Nys at 100,000 units per gram. UPLC and Franz diffusion cell instrumentation was used in the Crescent school of Pharmacy laboratory in Chennai (Tamil Nadu, India).

In vitro release testing (IVRT) method parameters

A laboratory validation method was applied to control the Nys and TA medication release from Nys and TA cream. The IVRT apparatus used was Franz diffusion cell instrumentation. This technique called for a 25 mm 0.45 µm Nylon membrane. The receptor media used in this technique consisted of water: tetrahydrofuran (50:50 v/v) [39]. The membrane was removed from the soaking medium and placed it over the bottom of the cavity of the sample chamber. The sample was applied on the membrane and spread with spatula to fill the entire cavity of the sample chamber the weight of sample about 300 mg [40]. The instrument was run with specified diffusion parameters and the specified volume of sample was withdrawn using suitable syringe or cannula from cell at each specified time intervals (such as 1, 2, 3, 4, 5, and 6 h) and changed at each point of time with the designated fresh preheated media volume using appropriate syringe (The material of construction used for the syringe is polypropylene plastic syringe with 2 mL capacity). Manual VDC was applied in every IVRT experiment to sustain a steady 32.0° ± 1.0 °C by means of the membrane temperature. At 500 rpm, the receptor medium inside the cells was stirred [41].

The Franz diffusion cell was used for In Vitro Release Testing (IVRT) with a 25 mm, 0.45 µm Nylon membrane serving as the diffusion barrier. A 50:50 v/v mixture of water and tetrahydrofuran functioned as the receptor medium. Prior to use, the membrane was soaked in the receptor medium and carefully placed in the sample chamber cavity.

Table 1 Optimized gradient program

Optimized gradient program		
Time (min)	**Mobile phase-A %**	**Mobile phase-B %**
0.0	98	02
1.0	98	02
6.0	50	50
7.0	98	02
9.0	98	02

UPLC method parameters

UPLC samples of Nys and TA were examined at wavelengths of 304 nm and 254 nm, respectively. For extraction, an aqueous mobile phase-A comprising 0.1% orthophosphoric acid in water, acetonitrile, and methanol in the ratio of 50:25:25 (v/v/v) and mobile phase-B consisting of acetonitrile and methanol in the ratio of 50:50 (v/v) was used, respectively. The 1.5 mL/min flow rate was chosen, using an Inertsil ODS-2 analytical column (50 mm x 3 mm, 5 µm size). Forty degrees Celsius was the column oven temperature. Extraction took place in a gradient environment; a 10 µL sample was injected for examination. Optimized gradient program is mentioned below in Table 1 [42].

UPLC method validation

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ values for Nys and TA were determined using signal to noise ratio as described in the literature [43]. To determine the LOD and LOQ for TA and Nys, the following approach was used. %RSD ≤ 2.0% at LOQ level indicates acceptable precision. LOD and LOQ were verified using the signal-to-noise ratio (S/N) method, as per ICH Q2(R1) guidelines on validation of analytical procedures.

- $LOD = 3.3 \times \sigma/S \ldots (1)$.
- $LOQ = 10 \times \sigma/S \ldots (2)$.

Where:

- σ = Standard deviation of the response.
- S = Slope of the calibration curve.

System suitability and specificity

The system suitability parameters were checked by injecting diluent (blank), standard solution, and calibration standards (CSs) into the chromatographic system. The retention times and the interferences were recorded [44].

Linearity

Linearity was performed for Nys standard in the range of 17.67-863.27 IU/mL and for TA standard in the range of 0.65–31.93 µg/mL [45]. The area response for each level was recorded and the slope, intercept, and regression coefficient (r^2) were calculated. The calibration curve was plotted by taking concentration IU/mL for Nys and µg/mL for TA on X-axis and area response on Y-axis [46]. Weighed and transferred about ~ 6.35 mg of TA and ~ 27 mg Nys into a clean and dry 50 mL volumetric flask, added 35 mL of methanol and sonicated to dissolve completely and volume made with methanol up to the mark. TA standard stock solution: 128.00 µg/mL and Nys standard stock solution: 3450.40 IU/mL.

Note TA stock concentration µg/mL (considering 100% potency),
Nys stock concentration (IU/mL) (considering 6418 IU per mg).

For solubilization, 100% methanol was used as solvent and dilutions were made in.

the water: tetrahydrofuran (50:50 v/v).

Preparation of linearity (calibration standards/range):

- CS-8: From the stock solution, taken 5 mL aliquot and diluted to 20 mL, yielding a final concentration of 32.00 µg/mL TA and 862.5 IU/mL Nys.
- CS-7: From the stock solution, taken 4.3 mL aliquot and diluted to 20 mL, yielding 27.52 µg/mL TA and 741.8 IU/mL Nys.
- CS-6: From the stock solution, taken 3.2 mL aliquot and diluted to 20 mL, yielding 20.48 µg/mL TA and 552.0 IU/mL Nys.
- CS-5: From the stock solution, taken 2.2 mL aliquot and diluted to 20 mL, yielding 14.08 µg/mL TA and 379.5 IU/mL Nys.
- CS-4: From the stock solution, taken 3.2 mL aliquot and diluted to 50 mL, yielding 8.19 µg/mL TA and 220.8 IU/mL Nys.
- CS-3: From the stock solution, taken 2.5 mL aliquot and diluted to 100 mL, yielding 3.20 µg/mL TA and 86.3 IU/mL Nys.
- CS-2: From CS-4 solution, taken 3.8mL (8.19 µg/mL TA and 220.83 IU/mL Nys) and diluted to 20 mL, yielding 1.56 µg/mL TA and 42.0 IU/mL Nys.
- CS-1: From CS-4 solution, taken 4 mL aliquot and diluted to 50 mL, yielding 0.66 µg/mL TA and 17.7 IU/mL Nys.

Accuracy and precision

Accuracy was performed by spiking lower limit of quantification (LLOQ), low-QC (LQC), middle-QC (MQC), high-QC (HQC), and upper limit of quantification (ULOQ) level standards to placebo of Nys and TA cream USP, 100,000 U/g; 0.1% solution in six preparations for each level. The accuracy at each QC level was calculated as % recovery. The precision was determined at intra-day and inter-day precision at LLOQ, LQC, MQC, HQC, ULOQ levels. The precision was expressed in terms of the percentage of relative standard deviation (% RSD) [47].

Weighed and transferred about ~ 6.35 mg of TA and ~ 27 mg Nys into a clean and dry 50 mL volumetric flask, added 35 mL of methanol and sonicated to dissolve completely and volume made with methanol up to the mark.

TA standard stock solution: 128.00 µg/mL and Nys standard stock solution: 3450.40 IU/mL.

Note TA stock concentration µg/mL (considering 100% potency),
Nys stock concentration (IU/mL) (considering 6418 IU per mg).

For solubilization, 100% methanol was used as solvent and dilutions were made in.

the water: tetrahydrofuran (50:50 v/v).

ULOQ: From the stock solution, taken 5 mL aliquot and diluted to 20 mL, yielding 32.0 µg/mL TA and 862.50IU/mL Nys.

- HQC: From the stock solution, taken 4 mL aliquot and diluted to 20 mL, yielding 25.60 µg/mL TA and 690.00 IU/mL Nys.
- MQC: From the stock solution, taken 4.5 mL aliquot was diluted to 50 mL, yielding 3.20 µg/mL TA and 310.50 IU/mL Nys.
- LQC: From MQC solution, taken 3.3 mL aliquot and diluted to 20 mL, yielding 1.90 µg/mL TA and 51.23 IU/mL Nys.
- LLOQ: From MQC solution, taken 3 mL aliquot and diluted to 50 mL, yielding 0.69 µg/mL TA and 18.63 IU/mL Nys.

Short-term working solution stability

The short-term working solution stability of TA and Nys was evaluated at ambient temperature. Six aliquots from freshly prepared working solution of TA and Nys

equivalent to CS1 and CS8 were kept on the work bench at ambient temperature [28]. After relevant stability period (initial and day-4), six standards of CS1 and CS8 were injected. The concentration of the TA (µg/mL) and Nys (IU/mL) from the stability standard solution equivalent to CS1 and CS8 was compared with concentration of the TA (µg/mL) and Nys (IU/mL) of the standard solution equivalent to CS1 and CS8 which are injected at each stability time period [19].

Long-term working solution stability

The long-term working solution stability of TA and Nys was evaluated at 2–8 °C temperature. Six aliquots from freshly prepared working solution of TA and Nys equivalent to CS1 and CS8 were kept in refrigerator at 2–8 °C [48]. After relevant stability period, (initial, and day-4) six standards of CS1 and CS8 were injected. The concentration of the TA (µg/mL) and Nys (IU/mL) from the stability standard solution equivalent to CS1 and CS8 was compared with concentration of the TA (µg/mL) and Nys (IU/mL) of the standard solution equivalent to CS1 and CS8 which are injected at each stability time period [49].

Validation of IVRT method

Following considerations guided the validation of the IVRT approach for Nys and TA in cream.

IVRT apparatus qualification

For IVRT equipment validation, the installation provided operational and performance qualification tools. Differsive area of the orifice, temperature of the receptor medium, and rotating speed (rpm) were assessed for equipment quality. Six cells were filled with receptor media, optimised membrane (0.45 µm Nylon membrane, 25 mm diameter) was placed in each donor cell and equilibrated for roughly twenty minutes before equilibration measurements were taken with a non-contact infrared thermometer, maintaining the temperature of the receptor media at 32 ± 0.5 °C. Temperature of the media was tracked when membrane was submerged in a cell with media [50].

IVRT membrane qualification

Membrane inertness was evaluated in relation to membrane binding of the Nys and TA in the receptor solution. Membranes were incubated in triplicate for the IVRT duration for 300 min at 32 °C ± 0.5 °C with the standard solution of Nys and TA prepared in receptor solution at 22.3 IU/mL and 603.1342 µg/mL. Three cells without membrane were run in parallel. The aliquots were collected before and after the duration of incubation. The samples were analysed by UPLC and the amount of Nys and TA from each aliquot with respect to the standard solution was calculated [51].

IVRT linearity

For Nys standard in the range of 17.67-863.12 IU/mL and for TA standard in the range of 0.65–31.80 µ/mL, linearity was performed. For every level, the area response was noted; slope, intercept, and r^2 were computed. Plotting concentration IU/mL for Nys and µg/mL for TA on X-axis and area response on Y-axis resulted in the calibration curve [52].

IVM linearity depends on the rate and extent of active metabolite release into the diffusion medium. All the collected sample intervals solutions shall be quantified by using the calibration curve of the respective analytes.

% Release = Area of collected sample at 1 h - Intercept of CC/slope of the CC.

Similarly calculated at all the time intervals. The calculated concentrations of all the time intervals was compared against the individual timepoints.

The IVRT linearity (regression coefficient (r^2) value) of the release rate (slope) was calculated across the range of the sampling times, which corresponds to the IVRT study duration. Linearity and range were demonstrated by performing IVRT study. Performed IVRT as per the method description and calculated the r^2 value between square root of time (in min) vs. corrected release of drug (in µg/cm^2).

IVRT recovery, mass balance, and dose depletion

The mass balance was characterized in each diffusion cell as accumulated (released) amount of Nys and TA in receptor solution over the IVRT duration and extracted amount of Nys and TA from the donor chamber remaining on the membrane at the end of the study [53]. Dose depletion was the amount of Nys and TA released from the formulation. The procedure of IVRT experiment and subsequent sample preparation was performed as described in method description [54]. The amount of Nys and TA released from the applied dose was calculated. For mass balance, the quantity released in the receptor medium and quantity remaining on the donor chamber of the membrane was evaluated [43, 55]. Calculations were done by comparing the extracted amount and released amount with respect to the applied amount of drug on each cell.

Robustness

The robustness of the Franz diffusion cell was evaluated by examining changes in the temperature, stirring speed, dosage amount, and composition of the receptor medium. Using Nys and TA cream, 0.05% as reference, the impact of temperature was compared to the standard conditions of 32.0 °C ± 5.0 °C. The method was considered robust to procedural modifications if the average slope of the IVRT run under altered conditions remained

within 15% of the average slope from the precision and reproducibility IVRT runs [48].

Results

UPLC validation

The UPLC validation method for Nys and TA encompassed assessments of system suitability, CS, specificity, linearity, precision, accuracy, stability, LOD, LOQ.

System suitability and CSs

The results of system suitability parameters are included in Table 2. It is determined that the system is appropriate for the quantification of Nys and TA in Nys and TA Cream USP, 100,000 U/g. For CSs, the % deviation for Nys and TA was less than 1%. The representative chromatograms of blank and TA are presented in Fig. 1.

Table 2 System suitability of TA and Nys

System suitability of TA and Nys		
Standard name	**TA-Area**	**Nys-Area**
Replicate-1	454359.501671	1901769.757933
Replicate-2	452428.418821	1896410.727696
Replicate-3	453762.235567	1901762.947938
Replicate-4	453919.524749	1902854.531971
Replicate-5	453747.074442	1901404.047164
Replicate-5	454441.819652	1904206.608155
Average standard area	453776.3	1901401.4
SD	724.07036	2651.56583
RSD (%)	0.2	0.1
USP tailing factor	1.1	1.2
USP plate count	12293	9452

The blank chromatogram did not show any peak of TA,

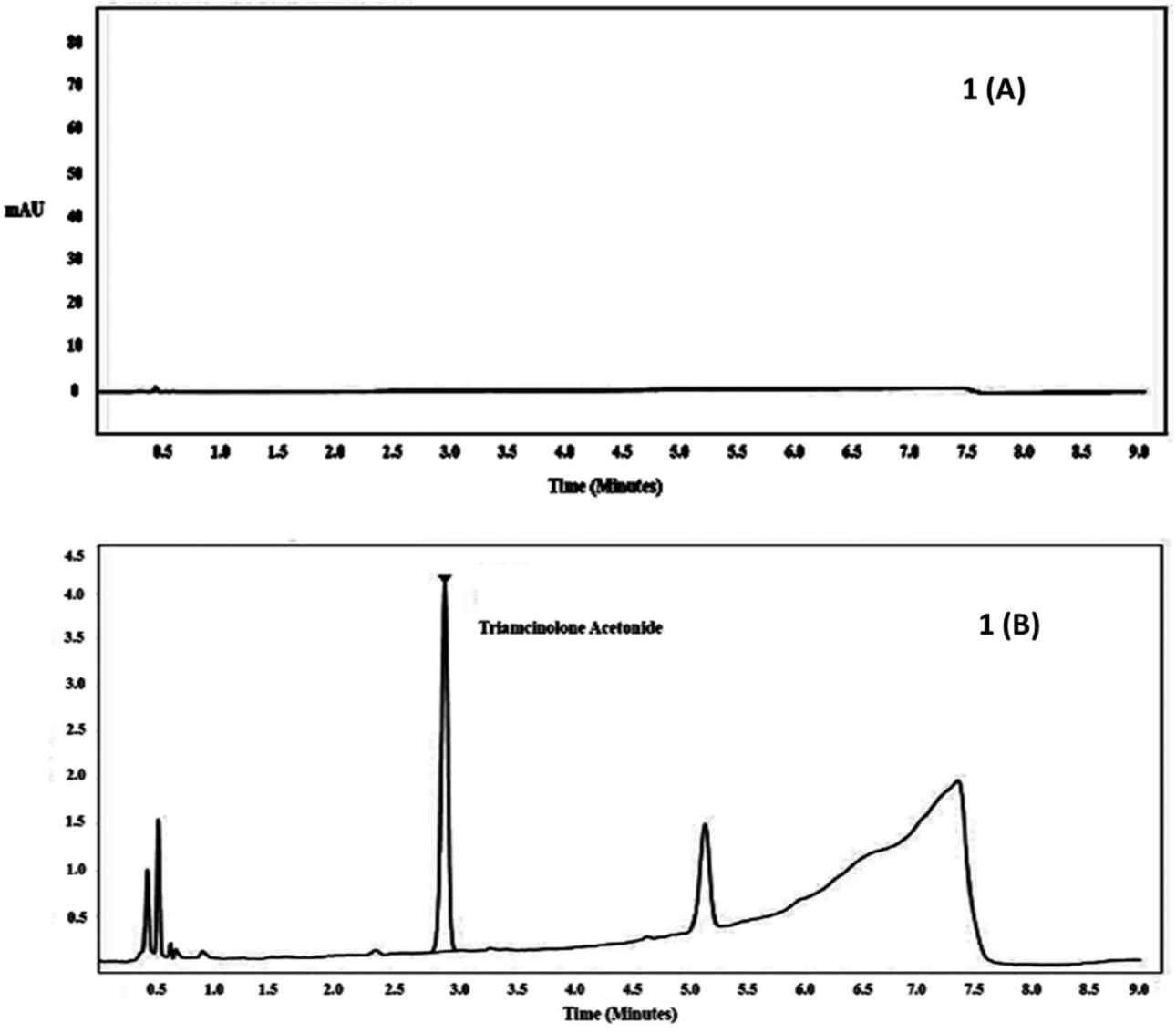

Fig. 1 Chromatograms of blank sample (**A**) and TA (**B**) recorded at 254 nm

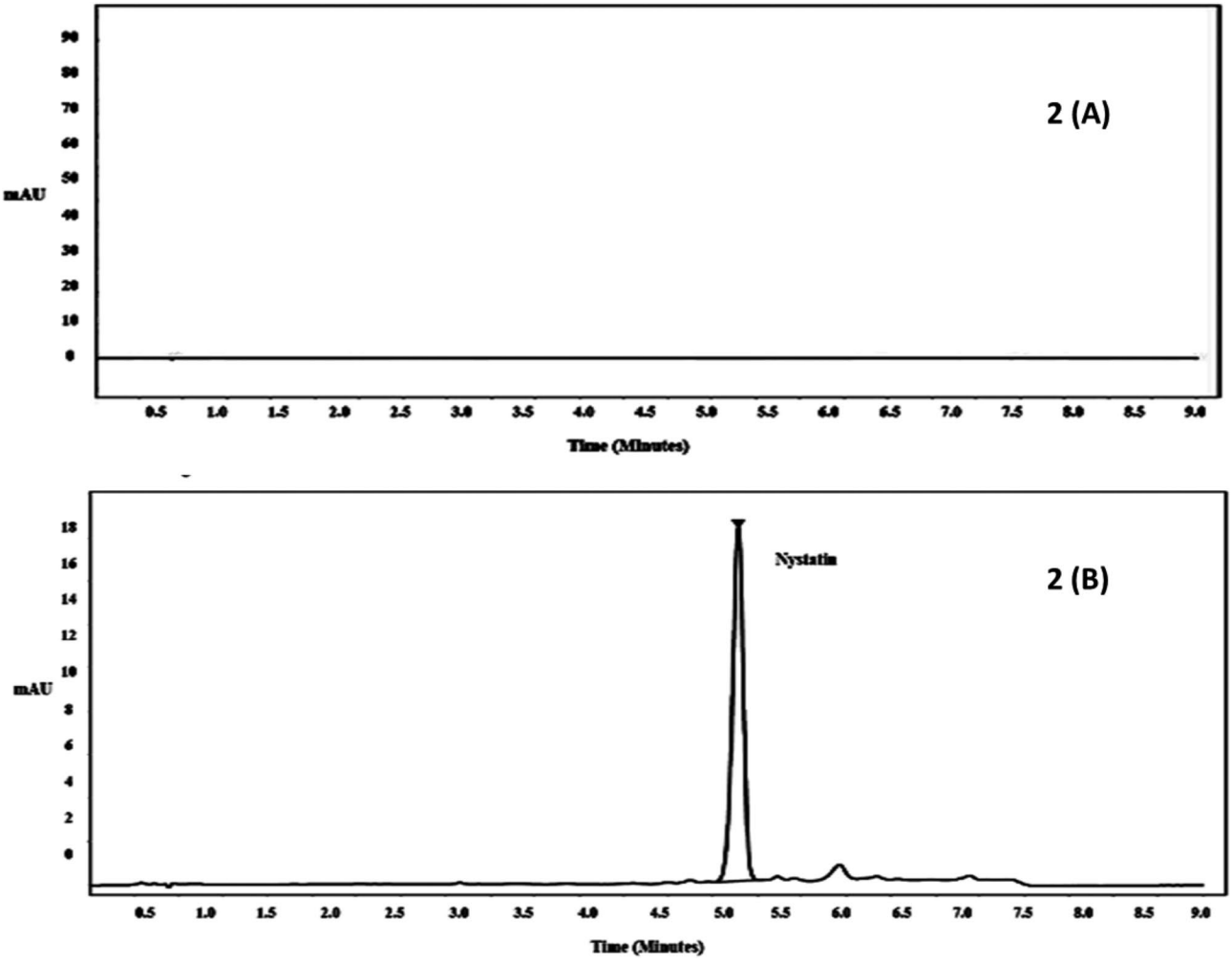

Fig. 2 Chromatograms of blank sample (**A**) and Nys (**B**) recorded at 304 nm

Table 3 Specificity of TA and Nys

Specificity (interference) for TA and Nys		
Name of injection	**Retention time of TA**	**Retention time of Nys**
Standard solution	~3.0	~5.0
Blank	No peak observed	No peak observed
Placebo	No peak observed	No peak observed

indicating no interference (Fig. 1A). The chromatographic peak of TA was found to be intact and sharp with acceptable chromatographic parameters (Fig. 1B).

The representative chromatograms of blank and Nys are presented in Fig. 2. The blank chromatogram did not show any peak of Nys, indicating no interference (Fig. 2A). The chromatographic peak of Nys was found to be intact and sharp with acceptable chromatographic parameters (Fig. 2B).

These findings suggested the suitability and CSs for the determination of Nys and TA in commercial creams using the proposed UPLC method.

Specificity

There was no interference observed from blank (diluent) and placebo at the retention time of Nys and TA (Table 3). These results indicated the specificity of the proposed UPLC method for the determination of Nys and TA.

We obtained the intercept values based on the range of the calibration curve, which compares the areas to the

Table 4 Linear regression data for the calibration curve of Nys and TA along with their LOD and LOQ data (mean ± SD, *n* = 6)

Parameters	TA	Nys
Linearity range	0.65–31.93 µg/mL	17.67–863.27 IU/mL
Regression equation	y = 39942x + 1753.8	y = 6198.9x + 12,007
r^2	1.0000	1.0000
Slope	39,942 ± 1012	6198.9 ± 103.0
Intercept	1753.8 ± 21.0	12,007 ± 411
Standard error of slope	584.29	59.46
Standard error of intercept	12.12	237.29
95% confidence interval of slope	36,727–41,756	5943–6454
95% confidence interval of intercept	1701–1805	10,986–13,028
LOD	0.12 ± 0.01 µg/mL	0.40 ± 0.02 IU/mL
LOQ	0.41 ± 0.03 µg/mL	1.33 ± 0.06 IU/mL

Table 5 Precision (% RSD) and accuracy (% recovery) data for Nys and TA using the proposed UPLC method (mean ± SD, *n* = 6)

Precision (% RSD) and accuracy (% recovery)								
Analyte	TA				Nys			
QC	Intra-day		Inter-day		Intra-day		Inter-day	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
LLOQ	100.6	0.4	99.8	0.5	99.4	0.1	99.6	0.1
LQC	99.7	0.2	102.4	0.7	100.4	0.1	102.3	0.6
MQC	99.5	0.2	100.9	0.2	100.2	0.2	100.9	0.2
HQC	99.7	0.1	101.2	0.1	100.1	0.1	101.1	0.0
ULOQ	99.7	0.1	99.6	0.1	99.6	0.1	99.5	0.1

respective concentrations. When, we plot the concentration against the area, we can calculate the intercept and slope. There is no interference from the blank in the calculation, as any blank interference was already checked in the specificity parameter and it met the acceptance criteria.

LOD and LOQ

The values of LOQ and LOQ for Nys and TA were calculated using signal to noise ratio and computed values are presented in Table 4. The LOD and LOQ for TA were found to be 0.12 and 0.41 µg/mL, respectively. However, the LOD and LOQ for Nys were derived to be 0.40 and 1.33 IU/mL, respectively. These results suggested that the proposed UPLC method was sensitive enough for the detection and quantification of Nys and TA.

Linearity

The results of linear regression analysis for Nys and TA are included in Table 3. From the linearity data, it is clear that the TA was found linear from 0.65 to 31.93 µg/mL and Nys was found linear from 17.67 to 863.27 IU/mL. The r^2 value for both medications was 1.0000. These results suggested the linearity of the proposed UPLC method for the determination of Nys and TA.

Accuracy and precision

To evaluate precision and accuracy over three batches, six sets of replicates were created for each of the QC level (LLOQ, LQC, MQC, HQC, and ULOQ), and results are summarized in Table 5. The intra-day and inter-day % recovery of Nys at different QC levels ranged from 99.4 to 102.3%. The intra-day and inter-day % recovery of TA at different QC levels ranged from 99.5 to 102.4%. The intra-day and inter-day % RSD of Nys at different QC levels ranged from 0.1 to 0.6%. The intra-day and inter-day % RSD of TA at different QC levels ranged from 0.1 to 0.7%. These findings led to the conclusion that the proposed UPLC method is accurate and precise for the determination of Nys and TA in commercial creams [45, 47].

Stability

This assessment provided insights into the stability of Nys and TA under different stress conditions. The stability results are summarized below:

- The short and long-term stock solution of Nys and TA was stable up to 4 days at 2–8 °C.
- Nys and TA and Nys working solutions (CS-1 and CS8) were stable up to 4 days at ambient temperature (25 °C) and at 2–8 °C.
- The Nys and TA solutions (LQC and HQC) were found stable up to 4 days at ambient temperature (25 °C), 2–8 °C, and − 20 °C.
- Nys and TA solutions (LQC and HQC) were found stable for 6 h at 34 °C.
- Nys and TA solutions (LQC and HQC) were found stable up to 4 freeze and thaw cycles at -20 °C.

Table 6 Linear regression data for the calibration curve of Nys and TA for IVRT linearity study (mean ± SD, *n* = 6)

Parameters	TA	Nys
Linearity range	0.65–31.80 µg/mL	17.67–863.12 IU/mL
Regression equation	y = 39365x + 559.8	y = 6223.7x + 5924.8
r^2	1.0000	1.0000
Slope	39,365 ± 1028	6223.7 ± 125.0
Intercept	559.8 ± 16.0	5924.8 ± 102.0
Standard error of slope	593.53	72.17
Standard error of intercept	9.23	58.89
95% confidence interval of slope	36,811–41,919	5913–6434
95% confidence interval of intercept	520–599	5671–6178

The precisions for each stability study was less than 2%. The accuracy for each stability study ranged from 93.0 to 105.3%. These findings indicated the stability of the proposed UPLC method for the determination of Nys and AT.

IVRT validation

Qualification of apparatus

Each parameter's results were in line with the predetermined acceptance standards for accuracy and precision. Specifically, the orifice diameter measured within 15.9 mm, the receptor medium temperature was maintained at 32.0° ± 0.5 °C, and the stirring speed was within ± 2% of the set RPM. These results demonstrated that, with all parameters falling within allowable bounds, the device is appropriate for IVRT method validation of Nys and TA in their combined cream formulation.

IVRT receptor solution sampling qualification

% RSD at three sampling point (initial, middle, and end six replicates) was found to be below 5.0% and % accuracy was 97.6% at initial, 98.9% at middle, and 98.4% at end sampling points which are found to be within 90.0-110.0% of nominal value. Hence, it was concluded that, the receptor solution sampling is suitable for IVRT of Nys and TA cream USP, 100,000 U/g; 0.1%.

IVRT linearity

The results of linear regression analysis are included in Table 6. For IVRT method, TA was found linear from 0.65 to 31.80 µg/mL and Nys was found linear from 17.67 to 863.12 IU/mL. The r^2 value for both medications was 1.0000. These results indicated the linearity of IVRT method.

Reproducibility

Reproducibility of the method was evaluated to check whether the method is reproducible or not. Each cell's r^2 value was determined to be ≥ 0.90, and the % RSD for the slopes of the six (intraday) and twelve (interday) cells was found to be within 15.0%. It was discovered that the cumulative % RSD for slopes of six and twelve cells (six of analyst-1 of day-1 and six of analyst-1 of day 2) was within 15.0%. Based on the aforementioned findings, it was determined that the approach was reproducible when carried out by one analyst on separate days.

IVRT recovery, mass balance and dose depletion

Dose Depletion was found to be in the range of 3.7 to 4.8% for TA and 13.8 to 15.7% for Nys. Mass balance for all six cells was found to be in the range of 96.7 to 103.4% for TA and 91.8 to 101.7% for Nys.

IVRT discrimination sensitivity, specificity, and selectivity

Sensitivity process is able to detect the changes in release of Nys and TA, as a function of different strengths of formulation. The amount of Nys and TA increases/decreases with increasing/decreasing Nys and TA concentration in formulation. Based on the results, it is demonstrated that the process is considered as sensitive.

The relationship between the formulation concentrations (50%, 100% and 150%) and the average IVRT release rate (slope) was carried out to determine IVRT specificity. The method was able to detect the changes in release of Nys and TA, as a function of different strengths of formulation. Based on the results, it was demonstrated that the method is specific.

Confidence interval obtained with respect to 50% vs. 100% at 8th term and 29th term was found to be 50.97 and 62.57 for TA, 54.21 and 58.39 for Nys and 150% vs. 100% was found to be 122.83% and 143.82% for TA, 142.22% and 163.24% for Nys and for altered formulation vs. 100% was found to be 45.28% and 51.21% for TA, 45.96% and 51.39% for Nys, respectively. From these results, it indicates that 50%, 150% concentrations and altered formulation were found to be in-equivalent with respect to the Nys and TA slopes from the test formulation (100% API) which demonstrates the selectivity of the IVRT method.

IVRT robustness

Robustness was evaluated for receptor medium composition variations, dose amount variations, variation in stirring speed, and temperature conditions. The results were found within the acceptable limits.

Discussion

The proposed UPLC method's validation for selectivity, specificity, linearity, precision, accuracy, stability, and robustness is supported by system suitability tests showing %RSD values of 0.2 and 0.1 for TA and Nys, respectively. The r^2 value was 1.0000 for each medication. This

validation aligns with the standards reported [55] by ensuring that our analytical method is highly sensitive and reliable for detecting low levels of active ingredients [22, 24]. The adherence to rigorous analytical standards confirms that our UPLC method is well-suited for accurate and precise measurement of Nys and TA, reinforcing its utility in pharmaceutical analysis and quality control [56–59].

In the IVRT methodology, the precise control over apparatus parameters, such as the diffusional area, receptor media temperature, and rotational speed, is fundamental for achieving reliable and reproducible drug release profiles. The study in the literature highlighted the critical role these parameters play in ensuring the consistency of drug release studies [39, 45, 47]. Our IVRT setup, featuring an orifice diameter of 15.9 mm, a receptor media temperature of 32 ± 0.5 °C, and a rotational speed maintained within ± 2% of 50 rpm, adheres to these recommendations [51]. This meticulous control mirrors the approach outlined demonstrating that our apparatus operates within the precision required for high-quality drug release testing [50]. The alignment with findings affirms that maintaining these variables within stringent limits is essential for producing accurate and reliable results [60, 61]. Another pivotal aspect of IVRT is membrane inertness. The choice of membrane material is crucial as it must not interfere with drug diffusion, which can lead to erroneous measurements. It has been emphasized the necessity of using inert membranes to prevent drug binding and subsequent inaccuracies in drug quantification [62]. Our study's results, which show no significant binding of drugs to the membrane over a 300-minute period, are consistent with these recommendations and further validated by Li and Lentz's protocols. This confirmation of membrane inertness ensures that our IVRT method remains accurate and reliable, avoiding potential sources of error that could compromise the integrity of the results [63]. The linearity of the IVRT method is another critical validation criterion. Our study demonstrated high linearity with r^2 value of 1.0000 for both medications, aligning with the results of the literature [42, 43]. This level of linearity indicates that our method is capable of accurately quantifying Nys and TA across a range of concentrations. The ability to maintain linearity across various formulation strengths enhances the method's versatility and reliability, making it suitable for diverse formulation analyses [64–65].

Recovery rates and mass balance are essential indicators of the method's accuracy. Our recovery rates for TA (3.7–4.8%) and Nys (13.8–15.7%) are consistent with the acceptable ranges reported by these results reflect the method's efficiency in recovering the drugs from the formulation and accurately measuring their quantities [7, 59]. The mass balance results, ranging from 96.7 to 103.4% for TA and 91.8–101.7%, while slightly higher, are within acceptable limits, suggesting that our method effectively accounts for the total amount of drug present. This comprehensive drug accounting further supports the robustness and accuracy of our IVRT methodology [66]. Reproducibility is a critical factor in validating any analytical method. Our IVRT method demonstrated reproducibility with %RSD for slopes within 15.0% and regression coefficients (r^2) ≥ 0.90. This finding aligns with the reported work which underscored the importance of minimal intraday and interday variability for ensuring reliable quality control [54]. The low variability observed in our study indicates that our IVRT method is consistent and reliable, suitable for routine application in quality control and formulation development [67]. The sensitivity and specificity of our method are further validated by comparisons with the results obtained in the literature [46]. Their use of similar techniques for detecting and quantifying active ingredients in topical formulations underscores the effectiveness of our method [68–71]. Our ability to differentiate between various formulation strengths and detect variations is crucial for both formulation development and regulatory submissions. This capability ensures that our method meets the high standards required for precise and accurate analysis of topical formulations. The robustness of our IVRT method is also notable. As demonstrated in the literature [53], slight variations in dose amount, receptor media composition, stirring speed, and temperature do not significantly impact the IVRT results. This finding validates the reliability of our method under varying testing conditions, highlighting its adaptability and consistency [63, 72, 73].

In summary, our comprehensive development and validation of IVRT and UPLC methodologies affirm their robustness and reliability. The alignment with existing research and the rigorous validation of our methods ensure that they meet high standards for accuracy, precision, and consistency. This reinforces their application in pharmaceutical analysis, contributing to effective formulation development and QC. The validation of IVRT apparatus and UPLC analytical method for Nys and TA in cream formulations underscore the robustness and reliability of these methodologies. Our study aligns closely with existing research, reinforcing the accuracy and precision of these testing techniques and contributing to the broader understanding of their application in pharmaceutical analysis.

Conclusion

This study concludes that the developed and validated analytical method for quantifying Nys and TA in topical creams demonstrate exceptional precision, accuracy, and robustness. The rigorous qualification of IVRT apparatus ensured meticulous control of key parameters

such as diffusional area, receptor media temperature, and rotational speed, aligning with stringent standards established in previous research, thereby guaranteeing consistent and reproducible drug release profiles. The evaluation of membrane inertness showed no significant binding of Nys and TA, confirming the reliability of the IVRT method. Excellent linearity, with r^2 of 1.0000, confirmed the method's capability to accurately measure a wide range of drug concentrations, ensuring robustness across different formulation strengths. Recovery rates and mass balance results within acceptable ranges validated the method's accuracy, confirming comprehensive drug accounting during the testing process. The reproducibility of the IVRT method, demonstrated by low intraday and interday variability, underscores its reliability for routine quality control and regulatory submissions. Additionally, the sensitivity and specificity of our method, comparable to results from similar studies, highlight its utility in distinguishing between different formulation strengths and variations, which is essential for detailed formulation analysis and development. The robustness of the IVRT method, demonstrated by its resistance to variations in dose amount, receptor media composition, stirring speed, and temperature, reinforces its reliability under various testing conditions. Furthermore, the validation of the UPLC method confirmed its high sensitivity and reliability for detecting low levels of active ingredients, with excellent selectivity, specificity, linearity, precision, accuracy, stability, and robustness. In conclusion, the extensive qualification and validation processes of the IVRT apparatus and UPLC analytical method confirm their suitability for the accurate and reliable quantification of Nys and TA in topical cream formulations, meeting all regulatory and scientific standards, and providing a robust framework for QC and regulatory assessment of topical formulations containing these active ingredients.

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Author contributions
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Data availability
"All data generated in this work will be made available from the reasonable request from corresponding author".

Declarations

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