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Original research article

Development and validation of an ultra performance liquid chromatography photodiode array method for quantification of low levels of triamcinolone acetonide in skin permeation studies

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ABSTRACT

An analytical method using ultra-performance liquid chromatography coupled with photodiode array (UPLC-PDA) was developed and validated to quantify triamcinolone acetonide (TA) at a low level in samples from skin permeation studies which were based in phosphate buffer solution. Dexamethasone was used as an internal standard (IS) for evaluation. The separation of analytes and IS was performed on an ACQUITY UPLCTM BEH Shield RP18 column (1.7 µm, 100 mm x 2.1 mm I.D.) with a gradient system using 0.05% trifluoroacetic acid and acetonitrile as mobile phases with 5 min total run time and a detectable wavelength of 242 nm for a maximum absorbance of TA. Calibration curve linearity was linear over concentrations ranging from 50 ng/mL to 1,000 ng/mL with a correlation coefficient (r^2) over 0.998. The lower limit of quantification (LLOQ) for TA was also as low as 50 ng/mL. Accuracy, precision, stability, and dilution integrity were evaluated to be within the acceptance criteria. Therefore, this newly developed UPLC-PDA method of high sensitivity and accuracy is promising for TA determination in samples from *in vitro* permeation experiments for dermatopharmacokinetic studies.

Keywords: triamcinolone acetonide, UPLC-PDA, dermatopharmacokinetics, topical drug delivery system

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1. Introduction

Triamcinolone acetonide (TA) is a synthetic corticosteroid compound with immuno-suppressive and anti-inflammatory properties. It was first approved by the Food and Drug Administration (FDA) in 1958 to treat various skin diseases such as eczema, dermatitis, allergies, rashes, psoriasis and allergic rhinitis.[1,2] TA can be formulated in many dosage forms, including cream, lotion, ointment, nasal spray, dental paste, and injectable solution. One of the most common clinical uses of TA is to treat atopic dermatitis, which is generally associated with disrupted skin barrier function increasing water loss. Moisturizers are then prescribed for the patients to improve skin hydration and relieve irritation together with the belief that they will also increase TA application efficiency through its greater skin permeation. However, there is no evidence concerning the impact of the order of application between moisturizers and topical TA on percutaneous absorption. Such a statement is also not specified in dermatology clinical practice guidelines. Only one study was found to investigate the sequential application of moisturizer lotions and azelaic acid gel *in vitro* using Franz diffusion cells and human trunk skin.[3]

To evaluate the influence of moisturizers on topical TA's percutaneous absorption, the amount of TA penetrating into and through the stratum corneum (i.e., the outermost layer of the skin) needs to be quantified. Several analytical methods, including high-performance liquid chromatography (HPLC),[3-10] ultra-high-performance liquid chromatography (UPLC)[11,12] and spectrophotometry,[13] have been reported with regard to determining TA alone or in combination with other compounds. However, there is no study addressing TA's simultaneous quantification at low level in phosphate-buffered saline (PBS) by an *in vitro* permeation study using human skin. Therefore, this present study aims to develop a practical and effective method using UPLC coupled with photodiode array detection (PDA) for quantification of TA in PBS at pH 7.4, and later in samples from the skin permeation study. Accuracy and efficiency of the developed protocol follow the Guidance for Industry, Bioanalytical Methods Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (USFDA CDER, 2018, BP).[14]

2. Materials and Methods

2.1 Instruments and analytical conditions

Acquity™ Ultra Performance Liquid Chromatography (Waters Corporation, Milford, MA, USA) was used for the separation module. Chromatographic separation was developed on an ACQUITY UPLCTM BEH Shield RP18 column (1.7 µm, 100 mm x 2.1 mm I.D., Waters Corporation, Milford, MA, USA). The mobile phase was pumped at a flow rate of 0.45 mL/min, and the column temperature was set at 50 ºC. A combination of acetonitrile (A) and 0.05% aqueous trifluoroacetic acid (v/v, B) was used as the mobile phase with an optimized gradient system of 30% A, 70% B for 3 min, 35% A, 65% B for 1 min and 30% A, 70% B for 1 min. ACQUITY UPLC® PDA Detector (Waters Corporation, Milford, MA, USA) was operated at wavelengths between 200-380 nm, and TA was quantified at a wavelength of 242 nm, based on its high sensitivity and specificity. The auto-sampler was conditioned at 8 ºC, and the injection volume was a full loop with 10 µL. Empower 2 software (Waters Corpo-ration, Milford, MA, USA) was used for data analysis.

2.2 Chemicals and reagents

TA (purity 98.4%) and dexamethasone (purity ≥ 98.0%, used as an internal standard, IS) were purchased from Sigma-Aldrich Ltd. (Steinheim, Germany), and their chemical structures are shown in Fig. 1. Acetonitrile, methanol and trifluoroacetic acid were HPLC grade and purchased from Labscan Ltd. (Bangkok, Thailand). Water used in the HPLC system was produced by a Milli-Q® water purification system (EMD Millipore, Billerica,

MA, USA). Other chemical used in this study were also of analytical grade. PBS of pH 7.4 was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.3 Preparation of standard solutions and calibration curve

TA and dexamethasone standards were accurately weighed and then prepared in methanol as stock solutions at concentrations of 5.04 mg/mL and 2.74 mg/mL, respectively. Subsequently, primary standard solutions of both were prepared by dilution of a stock standard solution with a mixture of methanol and water (50:50, v/v) to 100,000 ng/mL. Working standard solutions were prepared by dilution of primary standard solutions with a mixture of methanol and water (50:50, v/v) to concentrations of 1,250, 5,000, 10,000, 15,000, 20,000, and 25,000 ng/mL for TA, and 15,000 ng/mL for dexamethasone. Finally, six different concentration levels of calibration standards (CS) were prepared by spiking 20 µL of working standard solutions with 480 µL of PBS to final concentrations of 50, 200, 400, 600, 800, and 1,000 ng/mL. The quality control (QC) samples were prepared by dilution of primary standard solutions with a mixture of methanol and water (50:50, v/v) to concentrations of 3,750, 12,500, 22,500, and 30,000 ng/mL. The four different concentrations of quality control (QC) were prepared by spiking 20 µL of working standard solutions with 480 µL of PBS to create low, medium, high, and upper high controls at 150, 500, 900 and 1,200 ng/mL, respectively. All standard stock solutions, primary standard solutions, working solutions, and QC samples were stored at -20 °C until use.

2.4 Sample preparation

Twenty microliters of IS (dexamethasone at 15,000 ng/mL) were added with 0.5 mL of CS, QC and skin permeation samples into a 1.5 mL micro-centrifuge tube and mixed immediately. After that, 200 µL sample were diluted with 200 µL of Milli-Q water and mixed immediately. The samples were then centrifuged at 14,000 rpm for 10 min before collecting supernatants (200 µL) into a vial, which were later injected into the UPLC system.

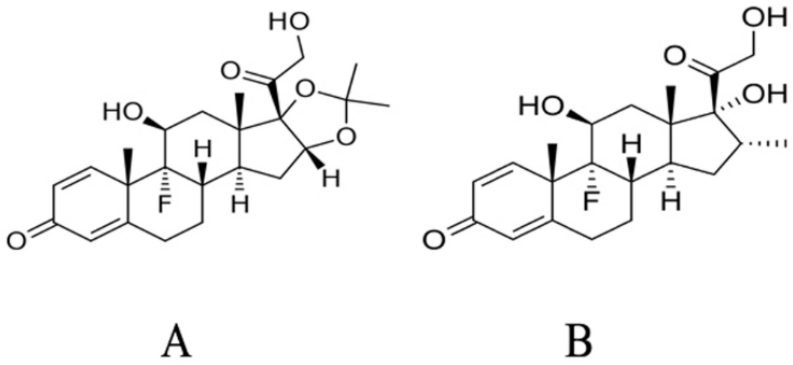

Fig. 1. Chemical structures of triamcinolone acetonide (A) and dexamethasone (B).

2.5 Method validation

The developed method was fully validated according to the United States Food and Drug Administration Guidance for Industry: Bio-analytical Method Validation; USFDA 2018.[14] A blank skin permeation sample in PBS at pH 7.4 was determined for specificity and observed to not have interfering peaks at the retention times of TA and IS. Sensitivity at the lower limit of quantification (LLOQ) was also determined using a dilution of TA working standard

solutions in PBS, and the lowest detectable concentration with a signal to noise ratio (s/n) more than five was defined as LLOQ. Accuracy and precision were evaluated using the QC samples at LLOQ and accepted when the values were within ± 20% by analyzing six replicates. At other concentrations (i.e., 150 ng/mL (low QC, LQC), 500 ng/mL (medium QC, MQC) and 900 ng/mL (high QC, HQC)), percentages of accuracy and precision were considered acceptable when being within ± 15% of nominal concentrations.

Calibration curves were obtained by plotting the areas under the curves (AUCs) against concentrations, and linear regression ($y = mx + b$; where y is the ratio between the peak area of the analyte and the peak area of IS, and x is the concentration of the sample at different levels including 50, 200, 400, 600, 800 and 1,000 ng/mL) was used to determine linearity. A correlation coefficient (r^2) of 0.995 or greater was considered acceptable.

Stability studies for auto-sampler, bench-top, freeze-thaw, stock solution, and long-term stability were evaluated using three replicates of QC samples at LQC, MQC, and HQC. The stability of each specific condition was calculated by comparing the samples quantity under the specific conditions to that of the freshly prepared samples. The accuracy was considered acceptable when being within ± 15%. A dilution integrity test was also performed using QC samples at an upper limit of quantification (ULOQ) of 1,200 ng/mL. These samples were serially diluted with PBS pH 7.4 to 600 ng/mL and then analyzed for at least five replicates. The accuracy and precision were required to be within ± 15%.

3. Results

3.1 Method development

The mobile phase compositions were optimized using a mixture of a standard solution of TA and IS. The isocratic program was optimized with the initial ratio of acetonitrile and water varying within a range of 30-50%. The aim was to determine the constituents and separate the co-eluting peaks. The isocratic condition with acetonitrile and water at a ratio of 30:70, v/v could separate the analytes, but this method required a long analytic time and led to peak tailing.

A gradient program was established for further modification. The initial ratio of acetonitrile was 30%, while the final ratio was set to 35%, allowing for elution of all peaks in a reasonable runtime of 5 min. Moreover, to improve the peak shape, trifluoroacetic acid (TFA) was added as an organic modifier in the water at 0.05% concentration. A final gradient was developed, in which the concentrations of TFA and acetonitrile were optimized to maximize the separation efficiency and minimize the time of the analysis.

The separation was achieved with a gradient program by 30:70 at 0 min, 35:65 at 3-4 min and 30:70 at 5 min. This method showed a good separation for TA and IS in 5 minutes, as shown in Fig. 2. System suitability parameters were calculated, and they were found to be excellent. The capacity factor (k′) was 3.89 for TA and 2.96 for IS; the resolution factor was 7.72. The ACQUITY UPLC® Photodiode Array (PDA) Detector from Waters Co. Ltd. (USA) was used to monitor a wavelength range of 200-380 nm. The PDA spectrum in Fig. 2 shows that the highest sensitivity for TA and IS was at 241.6 nm and 240.4 nm, respectively. However, the specific wavelength for this method was selected to be 242 nm for the measurement of TA. Wavelength selection was based on the requirement for high sensitivity and specificity for TA determination.

3.2 Assay performance and validation

3.2.1 Specificity and sensitivity

Six blank skin permeation samples were screened for interference of TA and IS. There were no interfering peaks for the retention time of TA and IS in specificity testing as shown in Fig. 3. The lower limit

of quantification (LLOQ) was 50 ng/mL, with a signal-to-noise ratio of 4.53 for TA determination. This method has acceptable precision and accuracy even though the signal-to-noise ratio was lower than 5. The precision at LLOQ was 6.11 %CV, and the accuracy was 108.52 %. Consequently, the developed method is selective and sensitive for TA and IS.

3.2.2 Linearity and calibration standard curve

The six-point calibration standard curve was linear over the concentration range of 50-1,000 ng/mL. The calibration standard curve was constructed by plotting TA to IS peak area ratio (y) against the TA concentration (x). This method showed good linearity with a determination coefficient (r^2) greater than 0.998 (0.9994, 0.9987, 0.9987 for three consecutive days), as shown in Fig. 4, which was within the acceptance criteria. The accuracy and precision were within ±20% at LLOQ and ±15% at other concentrations. The results are summarized in Table 1.

3.2.3 Accuracy and precision

The within-run and between-run accuracy and precision were examined by analyzing six replicates of LLOQ, LQC, MQC, and HQC for three consecutive days. Method accuracies of within-run and between-run were in the range of 95.85 to 104.20 % and 99.35 to 101.12%, respectively. The within-run and between-run precision was also determined, with %CV in the range of 1.23% to 7.19% and 1.89% to 5.90%, respectively. The results are summarized in Table 2. These results prove that the present method has precision and accuracy in determining TA in skin permeation samples.

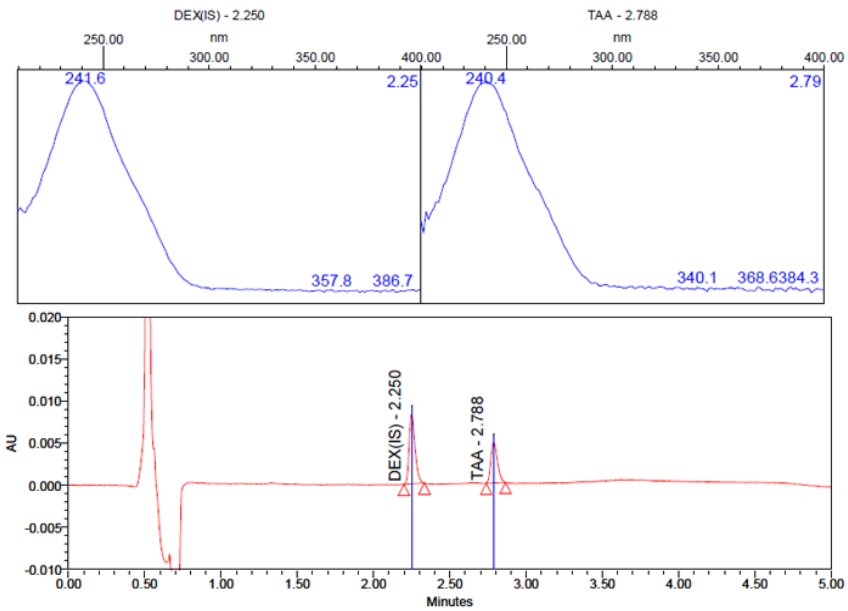

Fig. 2. The PDA spectrum of triamcinolone acetonide and dexamethasone.

3.2.4 Stability study

A simulated PBS test was performed to assess the stability of analysis spiked with three QC concentrations under various conditions compared with freshly prepared samples. TA has been proven to be stable in PBS pH 7.4 at the storage conditions specified for thawing stability (three cycles), bench-top stability (4 h at room temperature 25°C), long-term stability (90 days at -20°C), auto-sampler stability (4 h in an auto-sampler at 8°C) and stock solution stability (90 days at -20°C). The accuracy and precision were within an acceptable range of ±15%. The

results are summarized in Table 3. These results show that TA was stable in PBS pH 7.4 during the sample preparation and storage conditions.

Table 1. Precision and accuracy of Triamcinolone acetonide calibration standard curves.

Nominal concentration (ng/mL)	Measured concentrations[a] Mean±SD (ng/mL)	Precision (%)	Accuracy (%)
50	51.44±4.81	9.36	102.89
200	199.06±4.68	2.35	99.53
400	405.26±7.93	1.96	101.31
600	592.66±9.48	1.60	98.78
800	795.77±13.67	1.72	99.47
1000	1005.80±15.87	1.58	100.58

[a]Mean, n = 3 sets of calibration curves

Table 2. Within-run and between-run precision and accuracy study.

Nominal concentration (ng/mL)	Day	Measured value Mean ± SD (ng/mL)	Accuracy (%)		Precision (%)	
			Within run[b]	Between run[c]	Within run[b]	Between run[c]
50	1	51.25±2.45	102.50	99.35	4.78	5.90
	2	47.92±3.45	95.85		7.19	
	3	49.85±2.16	99.70		4.34	
150	1	151.05±6.00	100.70	100.61	3.97	3.90
	2	148.15±6.27	98.77		4.23	
	3	153.54±5.02	102.36		3.27	
500	1	498.68±13.94	99.74	99.36	2.79	1.89
	2	493.77±7.07	98.75		1.43	
	3	497.97±6.12	99.59		1.23	
900	1	937.79±44.60	104.20	101.12	4.76	3.62
	2	898.45±12.87	99.83		1.43	
	3	893.92±11.98	99.32		1.34	

[b] Six replicates (n=6) at each concentration.
[c] Three day runs (n=18) at each concentration.

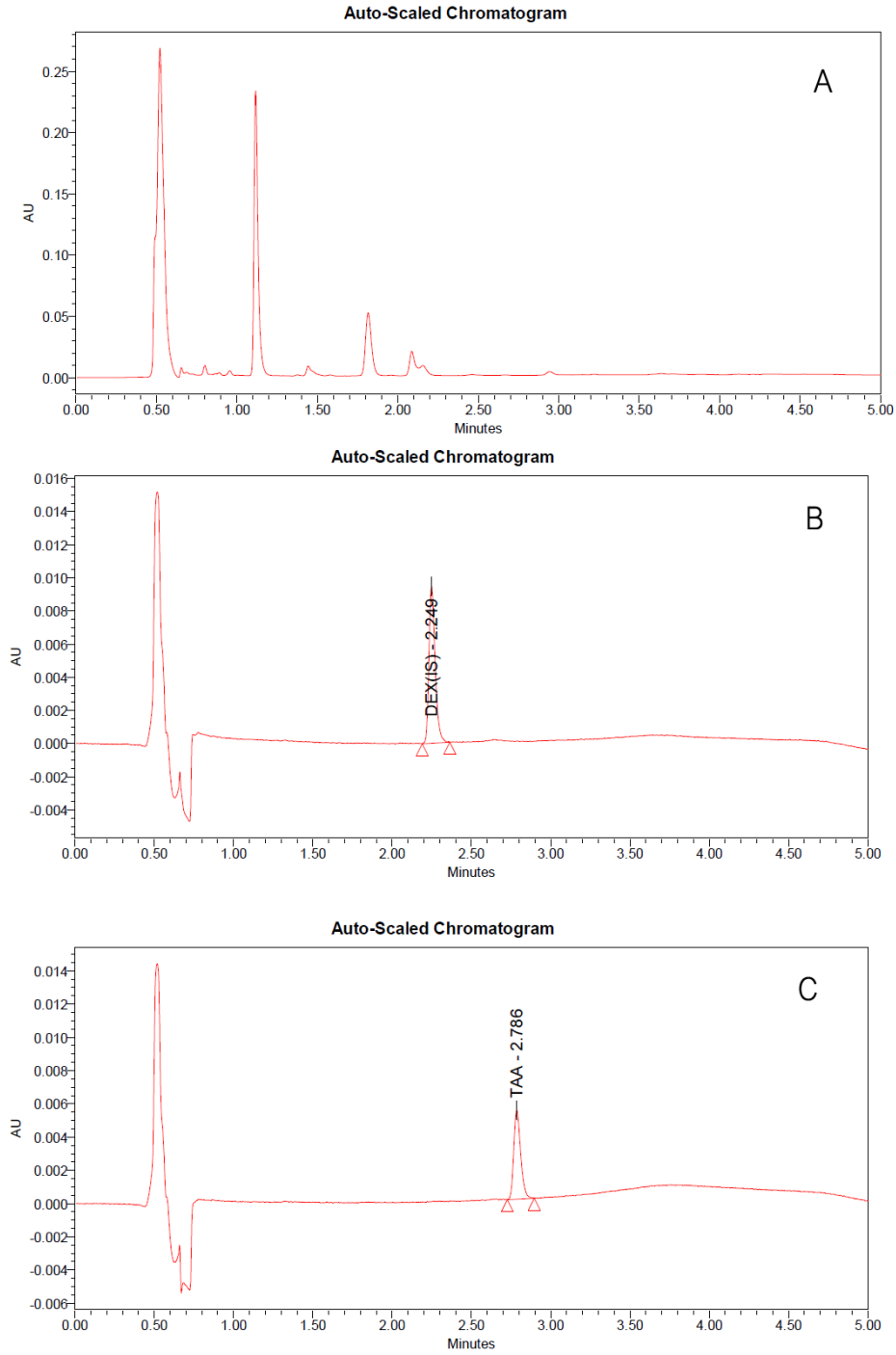

Fig. 3. The chromatograms of (A) blank skin permeation samples (B) PBS spiked with IS at 600 ng/mL (C) and PBS spiked with TAA at 600 ng/mL.

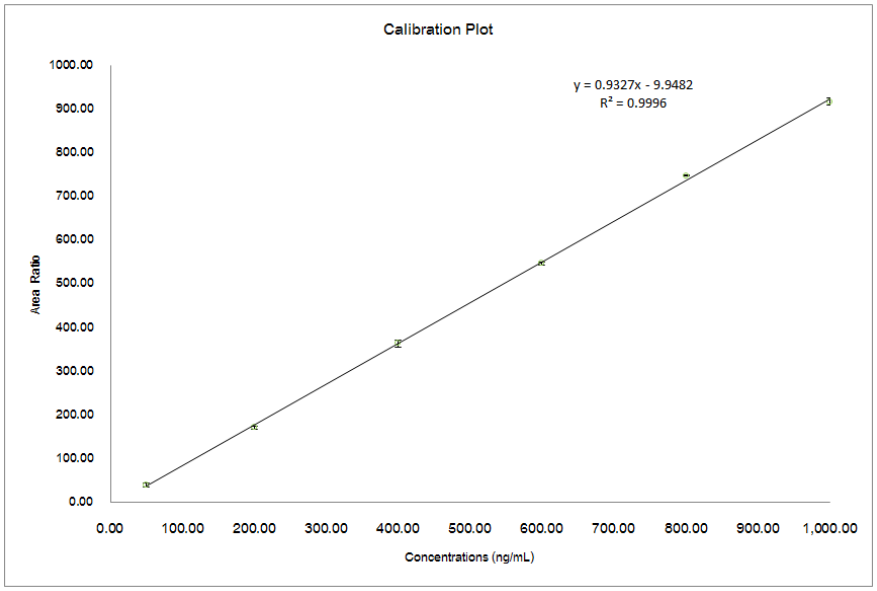

Fig. 4. The calibration curve for triamcinolone acetonide.

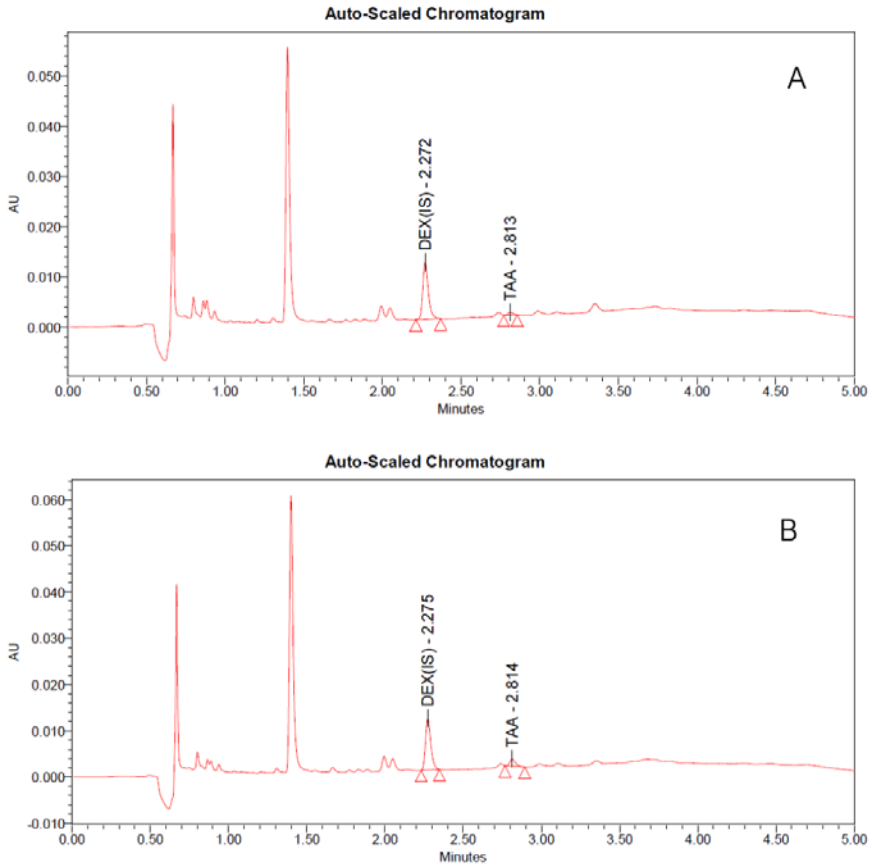

Fig. 5. Chromatograms of skin permeation samples at (A) 74.87 ng/mL and (B) 207.38 ng/mL

3.2.5 Dilution integrity

The dilution integrity was demonstrated by accurately measuring sample concentrations beyond the ULOQ range of 1,200 ng/mL with serial dilutions, as verified by six analyses. An accuracy of 93.10% was achieved and a CV of 2.66%, which is within an acceptable range.

3.2.6 Application

In the present study, a rapid and straightforward UPLC-PDA method for determining TA in PBS pH 7.4 was successfully developed. The method was fully validated and met all the requirements according to the USFDA guidance. This advanced method has been successfully used to quantify TA concentrations in the pilot study of moisturizer impact on percutaneous absorption of 0.1% TA cream, as shown in Fig. 5. The study was approved by the Siriraj Institutional Review Board [302/2558 (EC4)] of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Table 3. Stability of triamcinolone acetonide under various conditions.

Stability	Nominal concentration (ng/mL)	Precision (%)	Accuracy (%)	Differences (%)
Freeze and thaw stability (3 cycles, n=3)	150	2.26	100.11	-2.31
	500	0.29	101.28	1.50
	900	1.96	101.86	2.20
Bench-top stability (4 hrs., 25^{o}C, n=3)	150	0.54	98.09	-4.28
	500	2.54	99.33	-0.4
	900	0.80	99.94	0.28
Auto-sampler stability (4 hrs., 8^{o}C, n=3)	150	1.48	100.00	-0.17
	500	1.74	98.68	-0.57
	900	1.26	101.88	1.61
Long-term stability (90 days, –20^{o}C, n=3)	150	0.11	99.49	-2.91
	500	0.35	99.97	0.18
	900	0.65	100.21	0.55

n = number of replicates

4. Discussion

This study is the first report of a simultaneous quantitation of TA and dexamethasone by UPLC-PDA. The developed method was shown to be suitable for TA quantification in PBS from *in vitro* permeation samples using human skin. Compared to a high-performance liquid chromatography,[3-10] UPLC demonstrated lower retention times (5 min) and better chromatographic performance, leading to shortening analysis time and considerable savings in solvent consumption. The relative retention times were confirmed by injection of each standard and by comparison of their PDA spectra between 200 nm and 380 nm, which is easier than

methods such as mass spectrometry.[11-12] Moreover, these methods must show sufficient sensitivity, due to the low level of TA concentration in the skin permeation samples.

5. Conclusion

The developed UPLC-PDA method is simple, rapid, and reproducible for quantifying TA in PBS pH 7.4. It was fully validated and within the acceptance limit according to the USFDA standard guideline. It was also successfully applied to samples from *in vitro* permeation experiments for dermato-pharmacokinetic studies.

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