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A rapid HPLC method for simultaneous determination of tretinoin and isotretinoin in dermatological formulations

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Abstract

A rapid method using an isocratic high-pressure liquid chromatography and UV detection for determination of both all-*trans* retinoic acid (tretinoin) and 13-*cis* retinoic acid (isotretinoin) in dermatological preparations is presented. Tretinoin and isotretinoin samples were extracted with acetonitrile by a procedure that can be completed in less than 10 min. Subsequent separation and quantification of amounts as low as 10 pmol was accomplished in less than 15 min using reversed-phase HPLC with isocratic elution with 0.01% trifluoroacetic acid (TFA)/acetonitrile (15:85, v/v). Validation experiments confirmed the precision and accuracy of the method. When applied to commercial tretinoin samples, recoveries of 104.9% for cream formulations and 107.7% for gel formulations were obtained. Application of the method for analysis of a tretinoin cream exposed to solar simulated light (SSL) demonstrated detection of the major photoisomerization product isotretinoin as well as 9-*cis* retinoic acid, demonstrating the utility of the method for studies of tretinoin photostability. The method should also facilitate studies of the formulation compatibility and photocompatibility of tretinoin with agents that may improve its clinical tolerability.

Keywords: Tretinoin; Isotretinoin; HPLC; Photostability; Dermatological formulation

1. Introduction

Retinoids, a group of natural metabolites of Vitamin A (retinol) and related synthetic analogs, exert multiple biological effects in skin [1]. Retinoic acids with potent biological activity include the all-*trans* isomer (tretinoin), the 13-*cis* isomer (isotretinoin) and the 9-*cis* isomer (alitretinoin) [2]. Tretinoin is widely used for the treatment of a variety of skin conditions including acne [3] and photodamaged skin [4]. Methods that allow the rapid analysis of tretinoin and related compounds in dermatological formulations provide value from several standpoints. Tretinoin is relatively sensitive to heat and oxidation during storage and thus precise and accurate tretinoin quantification is important for quality control of finished products [5]. It is also quite photosensitive, leading to several degradation products, mainly isotretinoin [6], thus simultaneous determination of tretinoin and isotretinoin is important for studying factors that affect tretinoin photostability. While providing multiple benefits to skin [7], tretinoin therapy is frequently accompanied by significant skin irritation side effects [8]. Thus, improved methods of analysis also will facilitate studies of tretinoin compatibility with compounds that may improve its tolerability.

A number of previous methods for determination of tretinoin and/or isotretinoin have been described. The present analytical method in the United State Pharmacopoeia (USP) is a normal-phase HPLC system for analysis for isotretinoin in tretinoin products. Isotretinoin also has been determined by gas chromatography in soft and hard gelatin capsules [9]. Tretinoin has been determined in cream formulations by reversed-phase HPLC [5] and a reversed-phase HPLC method for determination of retinoids in pharmaceutical dosage forms with fluorescence detection has been described [10]. Although the above methods are able to separate and quantify tretinoin and isotretinoin, the extraction procedure is quite complex, and HPLC analysis involves long retention times. The chemical stability of tretinoin in dermatological lotions and hydrogels [11] and in hydrophilic gels [12] has been studied by simpler HPLC methods, but these

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methods do not report analysis of isotretinoin. HPLC separation methods for determination of tretinoin in ovules [13] and in solution [14] have been briefly described, and methods for analysis in biological samples have been reported [15,16]. The photostability of tretinoin lotion in the presence and absence of additives such as UV sunscreen, cyclodextrin and surfactants used an HPLC method with a gradient mobile phase composed of methanol–water–acetic acid mixture [17]. The photostability of tretinoin in niosomes as a carrier used an HPLC method that focused primarily on tretinoin in vesicular suspensions [18]. The photodegradation of tretinoin and isotretinoin in liposome formulations has been studied using spectrophotometric methods [19]. None of the methods described above have reported simultaneous determination of tretinoin and isotretinoin in dermatological formulations using a simple isocratic HPLC method.

We report here methodology that allows the rapid, precise and accurate determination of tretinoin and isotretinoin in gel and cream dermatological formulations. This method should be useful for determination of tretinoin and isotretinoin related to preformulation and formulation studies and for studies of the formulation compatibility and photocompatibility of potential agents to improve the tolerability of tretinoin therapy.

2. Experimental

2.1. Chemicals and reagents

Tretinoin, isotretinoin and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile (HPLC grade) was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). Brij-58, glyceryl monostearate, cetostearyl alcohol, white petrolatum, sorbic acid, butylated hydroxytoluene, simethicone, sorbitol 70% solution, propylene glycol and polyethylene glycol were purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA, USA). Tretinoin 0.025% cream was from Alpharma USPD Inc. (Baltimore, MD, USA). Tretinoin gel was from Clay-Park Labs Inc. (Bronx, NY, USA). Double distilled deionized water was used.

2.2. HPLC instrumentation and conditions

An HPLC system consisting of Varian Pro-star solvent delivery system model 230 (Varian Chromatography Systems, CA, USA), connected to a UV/Visible Spectroflow 757 absorbance detector (ABI, NJ, USA) and HP 3395 integrator (Hewlett Packard, DE, USA) was used for all separations. The injector was fitted with an injection loop of 50 μl. Chromatographic separations were performed using reversed-phase chromatography using a column of Phenomenex Nucleosil 5 μm C18 100 A, 250 mm × 4.6 mm (Phenomenex, CA, USA). The detection wavelength was 342 nm and sensitivity was set at 0.1 a.u.f.s. The mobile phase used was composed of 0.01% trifluoroacetic acid and acetonitrile (15:85, v/v%) at a flow rate of 1.0 ml/min. The mobile phase was filtered through a 0.45 μm membrane filter (Advantec MFS Inc., CA, USA) prior to use.

2.3. Preparation of dermatological formulations

Tretinoin and isotretinoin cream formulations contained: water, propylene glycol, sorbitol 70%, sorbic acid, butylated hydroxytoluene, simethicone, white petrolatum, cetostearyl alcohol, Brij-58, glyceryl monostearate, polyethylene glycol and tretinoin or isotretinoin. The ratio of oil phase to aqueous phase was (27.5:72.5, w/w). The water phase was mixed and placed in one container at 65–75 °C, and the oil phase was melted and mixed in another container at 65–75 °C. The oil phase was then added to the water phase and mixed until a cream was formed using an IKA mixer model RW 20DZM (IKA-works Inc., NC, USA). The cream was cooled to room temperature while stirring.

2.4. Extraction of dermatological formulations

A sample of 0.5 g of cream or gel was weighed into a 50 ml conical centrifuge tube using a Mettler balance model PB-303S (Mettler-Toledo, Switzerland). The sample was extracted by addition of 30 ml of acetonitrile followed by vortex mixing for 3 min. An aliquot of 1.5 ml was transferred to a microcentrifuge tube and subjected to centrifugation at 10,000 rpm for 5 min using an Eppendorf microcentrifuge (Brinkmann Inst. Inc., NY, USA). An aliquot of 50 μl of the supernatant was injected directly into the HPLC.

2.5. Quantification of tretinoin and isotretinoin

Stock solutions containing 1 mM of tretinoin or 1 mM isotretinoin were prepared in acetonitrile and diluted appropriately to provide standards for quantification. A standard curve was constructed by injecting samples containing tretinoin or isotretinoin at concentrations ranging from 0.0002 to 0.5 mM (0.01 to 25 nmol injected). The peak area was determined and plotted versus the concentration of tretinoin or isotretinoin. For the recovery studies, known volumes of tretinoin and isotretinoin standard solutions were analyzed, and the absolute recovery was calculated by comparing the peak area obtained from cream or gel with the peak area of samples derived from the standard solutions.

2.6. Exposure of tretinoin cream to solar simulated light (SSL)

A cream sample of tretinoin was exposed for 2 min to irradiation of SSL using a large area light source simulator, model 91293, from Oriel Corporation (Stratford, CT, USA) equipped with 1000 W Xenon lamp power supply model 68920. A sample of 0.5 g cream was weighed and extracted with acetonitrile as described above and then subjected to HPLC analysis.

3. Results and discussion

3.1. Development of conditions for rapid extraction and separation of tretinoin and isotretinoin from dermatological formulations

The desired goal of a method that would allow high throughput quantification of tretinoin and isotretinoin in dermatological

formulations was to complete an analysis from the formulation in a total of 30 min or less. The extraction procedure developed for tretinoin and isotretinoin from dermatological preparations allowed samples to be available for HPLC analysis in approximately 10 min. Conditions for a rapid and simple HPLC separation with UV detection were developed using an isocratic elution with a mobile phase composed of 0.01% trifluoroacetic acid and acetonitrile (15:85, v/v). These conditions gave well resolved, sharp peaks for both tretinoin and isotretinoin with retention times of approximately 11.3 min for tretinoin and 9.6 min for isotretinoin as shown in Fig. 1. Under these conditions, amounts of tretinoin and isotretinoin as low as 0.06 μg/ml could be readily detected. With these retention times, sequential analyses could be completed in less than 20 min. Since these conditions met our goal for high throughput analysis, validation experiments were completed to determine if the method could achieve the reproducibility and accuracy required for analysis of tretinoin formulations.

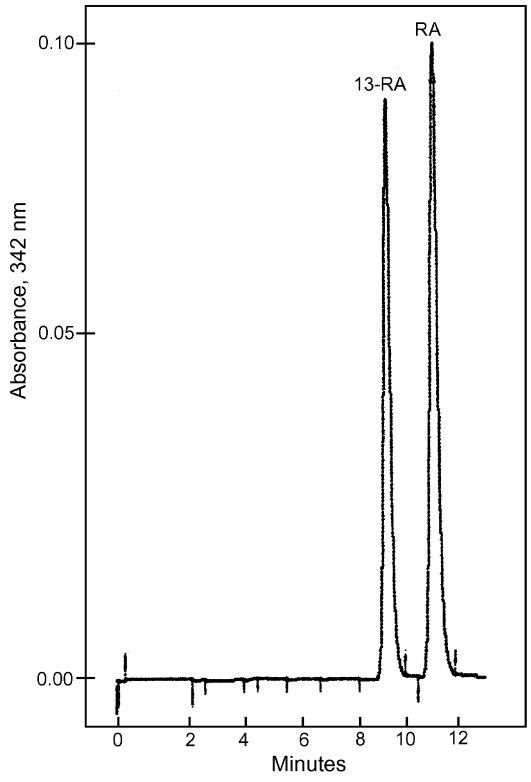

Fig. 1. Analysis by reversed-phase HPLC of a sample of a cream formulation containing tretinoin and isotretinoin. The formulation was extracted with acetonitrile as described in Section 2 and subjected to reversed-phase HPLC with isocratic elution with 0.01% trifluoroacetic acid (TFA) and acetonitrile (15/85, v/v) and UV detection at 342 nm. RA denotes tretinoin and 13RA denotes isotretinoin.

3.2. Method validation

3.2.1. Linearity

The quantification of the chromatogram was performed using the peak area of tretinoin and isotretinoin. Five standard solutions were prepared and subjected to triplicate analysis by HPLC. The peak area versus concentration was plotted in the concentration range from 0.0002 to 0.5 mM. Statistical analysis using least square regression analysis indicated excellent linearity for both tretinoin and isotretinoin in the range from 0.0002 to 0.50 mM as shown in Tables 1 and 2, respectively.

3.2.2. Accuracy and precision

The intra-day accuracy and precision of the assay was evaluated by analyzing five replicates of cream samples containing tretinoin and isotretinoin at three different concentrations as shown in Table 3. The intra-day precision of the analyzed samples as determined by R.S.D. (%) range from 0.51 to 1.46% which is within the acceptable range of 2%, while the intra-day accuracy of the method ranged from 99.1 to 104.3%. The inter-day precision of the assay was measured by analyzing replicates of 10, 25 and 50 mg% cream samples from day 1 to 3. Inter-day accuracy ranged from 102.9 to 108.9%, while the inter-day precision as determined by R.S.D. (%) ranged from 0.47 to 1.84% as shown in Table 4.

3.2.3. Recovery

The absolute recovery was calculated by comparing the peak areas of tretinoin and isotretinoin standards prepared in acetonitrile solutions to those obtained by extraction of cream or

Table 1
Statistical analysis of linear regression of tretinoin

Concentration (mM)	Peak area				
	1[a]	2[a]	3[a]	4[a]	5[a]
0.0002	17,234	17,146	16,983	17,573	17,398
0.001	86,173	87,560	84,987	85,732	87,234
0.005	372,678	374,568	373,467	372,358	373,638
0.01	1,049,720	1,037,252	1,056,103	1,055,804	1,057,892
0.05	3,749,614	3,767,824	3,736,549	3,744,469	3,756,453
0.1	7,126,941	7,183,392	7,081,443	7,115,987	7,103,456
0.25	18,859,350	18,240,500	18,638,456	18,556,890	18,478,934
0.5	37,718,700	37,823,456	37,598,764	37,686,740	37,805,678
Intercept	−4252	−37,194	−14,325	−19,589	−31,728
Slope	7.53E + 07	7.51E + 07	7.50E + 07	7.51E + 07	7.52E + 07
R^2-value	0.9998	0.9996	0.9998	0.9997	0.9997

[a] Run no.

Table 2
Statistical analysis of linear regression of isotretinoin

Concentration (mM)	Peak area				
	1[a]	2[a]	3[a]	4[a]	5[a]
0.0002	19,417	19,600	19,474	19,598	19,389
0.001	114,255	119,370	112,372	115,723	116,578
0.005	572,275	573,452	570,986	574,396	571,328
0.01	407,619	428,027	399,353	408,965	395,479
0.05	3,277,633	3,312,453	3,294,704	3,225,742	3,305,646
0.1	6,315,614	6,357,629	6,341,658	6,247,555	6,322,374
0.25	15,513,371	15,565,952	15,586,032	15,388,128	15,522,546
0.5	31,028,473	31,145,682	31,263,258	31,325,637	31,197,638
Intercept	62,613	72,574	52,959	17,033	53,755
Slope	6.19E + 07	6.21E + 07	6.24E + 07	6.24E + 07	6.22E + 07
R^2-value	0.9998	0.9998	0.9990	0.9990	0.9990

[a] Run no.

Table 3
Intra-day accuracy, precision and relative error of quality control samples of tretinoin and isotretinoin

	Theoretical (mg%)	Found (mg%)	R.S.D. (%)	Accuracy (%)	R.E. (%)
Tretinoin	10	10.31	0.51	103.1	3.10
	25	25.24	0.83	100.9	0.96
	50	49.54	1.46	99.1	−0.92
Mean				101.0	
Isotretinoin	10	10.43	0.63	104.3	4.30
	25	25.26	1.02	101.0	1.04
	50	50.47	1.37	100.9	0.94
Mean				102.1	

$N = 5$.

gel preparations. The results of absolute recoveries of tretinoin and isotretinoin cream ranged from 97.6 to 107.3% as shown in Table 5. These recoveries are within range of the USP Monograph for tretinoin creams and gels.

3.3. Application of method to commercial tretinoin preparations

In order to test the method on commercial products, the absolute recoveries of tretinoin from Tretinoin Cream® 0.025% and Tretinoin Gel® 0.025% commercial products was determined as shown in Table 6. The results indicate excellent recovery from both cream and gel preparations that fall within limits of the USP Monograph.

Table 4
Inter-day accuracy, precision and relative error of quality control samples of tretinoin and isotretinoin

	Theoretical (mg%)	Found (mg%)	R.S.D. (%)	Accuracy (%)	R.E. (%)
Tretinoin	10	10.47	0.47	104.7	4.70
	25	26.12	1.12	104.5	4.48
	50	51.84	1.84	103.7	3.68
Mean				104.3	
Isotretinoin	10	10.89	0.89	108.9	8.90
	25	26.37	1.37	105.5	5.48
	50	51.46	1.46	102.9	2.92
Mean				105.7	

$N = 5$.

Table 5
Absolute recovery of tretinoin and isotretinoin from cream preparations

	Concentration (g%)	Peak area		Recovery (%)
		Acetonitrile	Cream	
Tretinoin	0.01	489,760	478,073	97.6
	0.025	1,134,198	1,175,194	103.6
	0.05	2,176,574	2,335,496	107.3
Mean				102.8
Isotretinoin	0.01	349,012	371,679	106.5
	0.025	894,532	926,757	103.6
	0.05	1,802,065	1,873,463	103.0
Mean				104.3

$N = 5$.

3.4. Application of method to studies of tretinoin photostability

An example of the application of the method for the study of photodegradation of tretinoin in topical dermatological cream is shown in Fig. 2. Panel A shows that the formulation of tretinoin not exposed to SSL did not contain detectable amounts of isotretinoin. However, Panel B shows that following SSL exposure for 2 min, the appearance of isotretinoin can clearly be

Table 6
Absolute recovery of tretinoin from cream and gel commercial tretinoin products

	Concentration (g%)	Peak area		Recovery (%)
		Acetonitrile	Cream	
Tretinoin cream	0.025	1, 134, 198	1,190,014	104.9 ± 1.3
Tretinoin gel	0.025	894, 532	963,612	107.7 ± 1.7

$N = 5$.

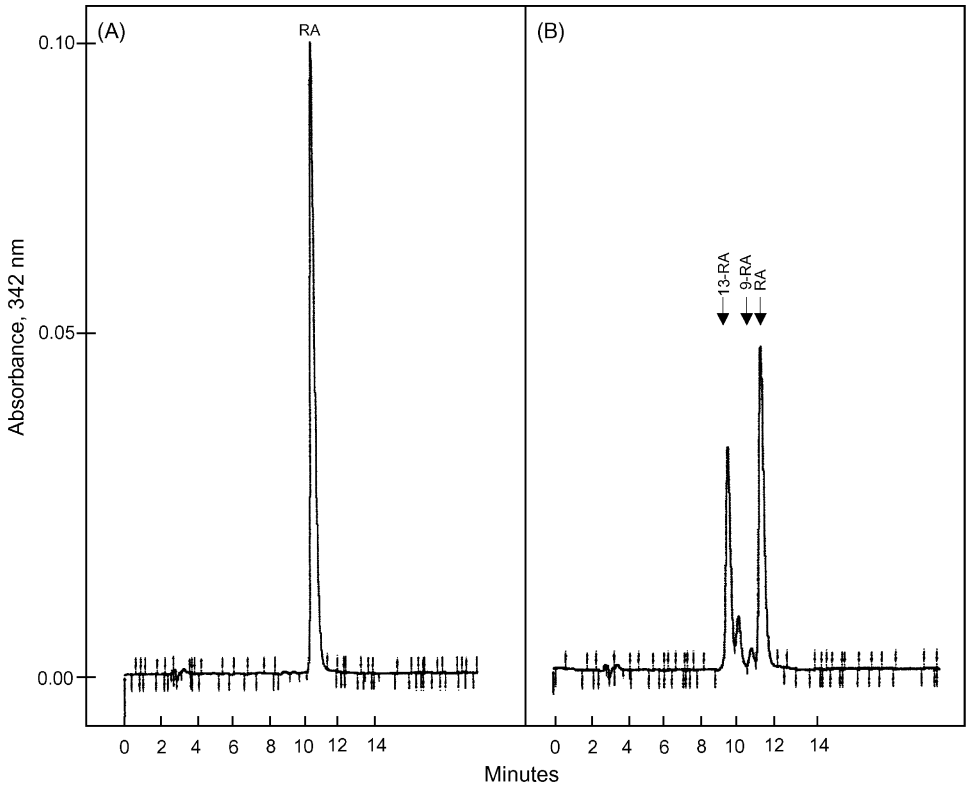

Fig. 2. Application of the method for analysis of a cream preparation subjected to solar simulated light (SSL). Panel A shows a tretinoin sample not exposed to SSL and Panel B shows a sample exposed to SSL for 2 min. RA denotes tretinoin, 13 RA denotes isotretinoin and 9 RA denotes 9-*cis* retinoic acid.

detected. Additionally, smaller amounts of two additional products were detected. We tentatively identified 9-*cis* retinoic acid as one of the products by spiking a solution sample of tretinoin and isotretinoin with 9-*cis* retinoic acid. The other product detected remains unidentified. However, neither product interfered with the quantification of tretinoin or isotretinoin despite the rapid analysis time.

4. Conclusions

The method described here allows the accurate determination of tretinoin and isotretinoin in dermatological formulation is as little as 30 min. The method can be applied to analysis of commercial tretinoin formulations. Tretinoin is an important drug that provides benefit to dermatology conditions that range from skin photodamage to acne. Rapid methods of analysis will facilitate preformulation and formulation studies of tretinoin topical formulations and will be useful for studies designed to lead to the discovery of agents that can enhance the tolerability of this important therapeutic agent. However, the method as currently configured would not qualify as a stability indicating method, particularly for photochemical stability, as the separation parameters between tretinoin and the minor photoproducts observed would need to be improved to achieve greater separation. However, it is envisioned that straightforward modifications of the mobile phase could achieve the degree of separation needed for applications where a stability indicating method would be required.

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