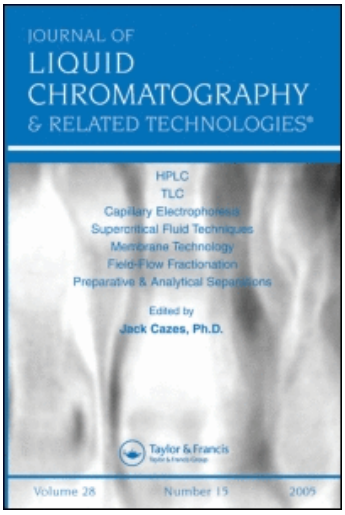

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Quantitation of Doretinel in a Topical Gel Using High Performance Liquid Chromatography with Solid Phase Extraction Sample Clean up

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QUANTITATION OF DORETINEL IN A TOPICAL GEL USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH SOLID PHASE EXTRACTION SAMPLE CLEAN UP

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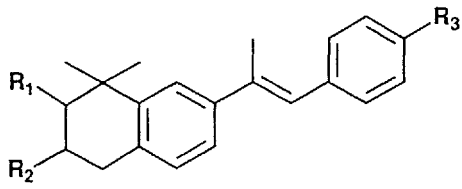
ABSTRACT

A stability-indicating method based on high performance liquid chromatography has been developed for the quantitation of doretinel, a new retinoid, at sub-ppm levels in an alcohol gel formulation. A high molecular weight polymer used as a thickening agent in the formulation is removed by solid phase extraction on a C_{18} column to permit large injection volumes. Samples are assayed on a reversed-phase column packed with 5 micron Spherisorb ODS-II and a mobile phase containing 50% methanol and 10% tetrahydrofuran in a 0.025M potassium phosphate solution. Separations are obtained under ambient conditions with an eluant flow rate of 1.5 mL/min. Placebo interferences are minimized by choosing 300 nm as the detection wavelength. Quantitation is by external standard. Linearity over a wide concentration range is observed. Relative standard deviations for precision and accuracy are less than 3%. Average recovery was 98.4% of theoretical: regression analysis of the data yielded a recovery of 100%.

INTRODUCTION

Doretinel (I) is a member of a class of compounds known collectively as retinoids. The important physiological function they perform in nutrition and vision has been recognized for decades. Recent research on this class of compounds has shown them to be mediators of cell differentiation and proliferation.[1] Thus, the domain of research in retinoids has moved beyond its classical roots in nutrition into such diverse areas as cancer treatment[2] and the treatment of skin damage caused by sun exposure.[3] This particular compound is being studied for its potential use as a dermatological product.

Increased retinoid biological research has, by necessity, been paced by an increased ability to perform quantitative analysis on these compounds. A wealth of literature is published on the HPLC analysis of retinoid compounds, particularly in complex biological matrices. Much of the literature describes methods using complex sample preparation schemes, i.e, lyophilization and extraction[4] or complex chromatographic conditions i.e., normal-phase chromatography, column switching and gradient-elution.[5] The goal of the project reported here was to develop a simple, rapid, isocratic, reversed-phase assay method for doretinel that was suitable for release, uniformity and stability assays in gel samples containing as little as 1 ppm of doretinel. Particularly challenging was the presence of a high molecular weight formulation excipient that caused HPLC system failure, due to excessive back pressure, when introduced into the column. This paper reports on a relatively simple, fast HPLC method with adequate sensitivity, specificity and precision for performing the required assays.



I $R_1 = OH, R_2 = H, R_3 = CH_2OH$
II $R_1 = O =, R_2 = H, R_3 = COOH$
III $R_1 = H, R_2 = OH, R_3 = CH_2OH$
IV $R_1 = O =, R_2 = H, R_3 = COOC_2H_5$

MATERIALS AND METHODS

Equipment: The liquid chromatographic system consists of a Waters (Milford MA.) isocratic HPLC equipped with a 590 pump and a WISP 712 autoinjector, an ABI Applied Biosystems Spectroflow 783 variable wavelength detector (Foster City, CA) and a Hewlett Packard (Piscataway, N.J.) 359000 dual channel interface. A Hewlett Packard 1040A Photodiode array was used during method validation but is not used for routine assays. A Spherisorb ODS-II, 5 micron column (150 x 4.6 mm I.D.), is used as the stationary phase. (Alltech, Deerfield, IL.) The data are collected and stored using a Hewlett Packard 1000/3350A laboratory automation system. Sample preparation utilizes Waters Sep-Pak^R C_{18} cartridges and 10 cc disposable syringes (Becton Dickinson, Rutherford, N.J.).

Chemicals and Reagents: All chemicals and reagents used are ACS Reagent Grade. The water, methanol, and tetrahydrofuran are HPLC grade. Reference standard material for doretinel was obtained in-house. Authentic samples of synthesis related compounds were obtained from BASF Aktiengesellschaft (Ludwigschaffen, West Germany).

Formulation: The topical gel formulation consists of specially denatured alcohol, hydroxypropyl cellulose, butylated hydroxytoluene and 1 to 10 ppm of doretinel.

Chromatographic Conditions: The HPLC system employs a column packed with 5 micron Spherisorb ODS-II. Doretinel is eluted from the column with a mobile phase containing 50% methanol and 10% tetrahydrofuran in a 0.025M potassium phosphate solution. Separations are obtained under ambient conditions with an eluant flow rate of 1.5 mL/min. A 500 μL aliquot of a solid-phase extracted sample is injected into the chromatograph. Quantitation is against an external standard at 300 nm.

Mobile Phase Preparation: The aqueous portion of the mobile phase is prepared by weighing 3.4 $\pm$ 0.1 g of potassium dihydrogen phosphate into a 1000 mL volumetric flask, which is dissolved and diluted to volume with water. The mobile phase is prepared by mixing 500 mL of methanol with 100 mL of tetrahydrofuran in a 1000 mL volumetric flask. This is diluted to volume with the 0.025M phosphate solution. After mixing, dissolved gasses are removed by placing the mobile phase under vacuum in an ultrasonic bath for 10 minutes.

Preparation of the Reference Standard: Approximately 12 mg of doretinel reference standard is accurately weighed and transferred to a 250 mL volumetric flask. The drug is dissolved in 10 mL of methanol and diluted to volume with the mobile phase to obtain the stock solution. Ten mL of the stock solution is diluted to 100 mL with mobile phase to obtain an intermediate solution. Ten mL of the intermediate solution is diluted to 250 mL with mobile phase to produce an analytical reference standard solution having an approximate concentration of 0.2 μg/mL of doretinel.

Assay Procedure: A 1.00 - 2.00 g sample of doretinel gel is accurately weighed into the barrel of a 10 cc disposable syringe. A Sep-Pak^{R} C_{18} cartridge, which has been prepared for use by rinsing with 2 mL of methanol followed by 10 mL of water, is inserted onto the end of the disposable syringe. Approximately 5 mL of water is added and the syringe plunger is inserted just enough to stopper the syringe opening. The syringe is vortexed to break up the gel and the gel is pushed through the cartridge. An 8 mL aliquot of water is added (with additional vortexing, if necessary) and pushed through the cartridge. This is repeated until all the water soluble excipients are eluted from the cartridge which is signaled by an end to the eluant foaming. Approximately 5-6 rinses or 50 mL of water are required. Remaining components, including the doretinel, are eluted from the cartridge into a volumetric flask with 1-5 mL of tetrahydrofuran followed by a 5 mL methanol rinse. The sample is diluted to volume with an appropriate mix of solvents to mimic the mobile phase. Sample weight and final dilution volume are chosen so that the concentration of doretinel in the final sample is approximately 0.2 μg/mL.

System Suitability: The retention time and area reproducibility of the doretinel peak with a minimum of five consecutive injections of the reference standard must be less than 2.0% RSD. The retention time window for the doretinel peak is 10 $\pm$ 1.25 minutes. The capacity factor for the doretinel peak must be greater than 10 and the peak asymmetry factor must be less than 1.3

RESULTS AND DISCUSSION

Previously developed methods in our laboratories for the determination of doretinel in alcohol gels were suitable

for concentrations greater than 50 ppm. These methods used dilute and shoot sample preparation procedures, small injection volumes and UV detection at 220 nm. These methods for 1 ppm samples were unsuitable for several reasons: inadequate sensitivity with small injection volumes, immediate system failure with large injection volumes due to high molecular weight excipients in the formulation, and severe placebo interferences at 220 nm. Method development efforts concentrated on sample clean-up and increased method specificity. Attempts at separating the hydroxypropyl cellulose based on salt-gradient and pH controlled liquid-liquid extraction were unsuccessful. Based on recent methods describing the use of solid-phase extraction[6] and the limited water-solubility of doretinel, a solid-phase extraction on a C_{18} stationary phase using water to remove the polymer was investigated. This approach did permit larger injection volumes, but, as the sensitivity required to detect the low levels of drug was achieved, the remaining excipients severely interfered with the doretinel quantitation. By choosing 300 nm as the wavelength of detection, these interferences were eliminated. An example chromatogram for a 1 ppm sample is displayed in Figure 1.

Specificity

The specificity of the method was tested by injecting authentic samples of synthesis-related compounds. Table I lists the observed retention time, relative retention time, capacity factor and resolution for these samples. All compounds were found to be baseline resolved from the doretinel.

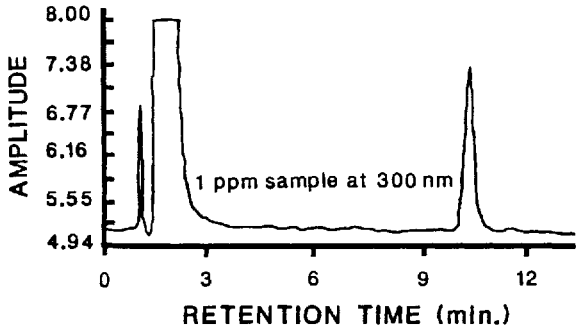


Figure 1: Example chromatogram of a gel sample containing 1 ppm doretinel.

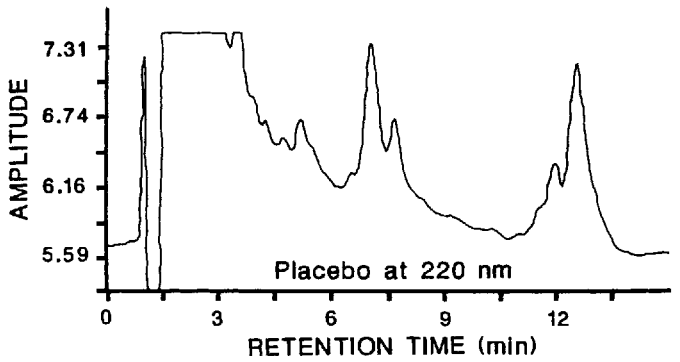


Figure 2: Chromatographic profile of placebo at 200 nm. Interference from excipients requires use of a more selective detection wavelength.

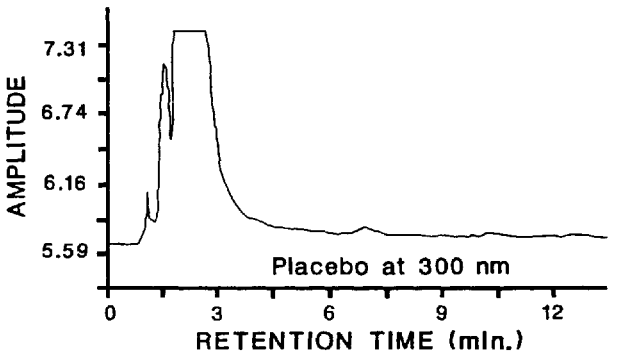


Figure 3: Chromatographic profile of placebo at 300 nm. Interference is minimized and low-level quantitation of doretinel is possible.

Specificity of the method with respect to placebo samples was shown by preparing placebo samples by solid-phase extraction and examining their chromatographic response. At 200 nm, placebo interferences were too severe to permit quantitation of doretinel (Figure 2), while at 300 nm (Figure 3), there are no interfering peaks eluting in the time interval of interest.

Stability Indicating Ability

The stability indicating properties of the method were tested through chromatographic examination of doretinel samples subjected to various conditions of stress such as acid hydrolysis (2 days at 70°C in 0.3N HCl), base hydrolysis (2 days at 70°C in 0.3N NaOH), chemical oxidation (m-chloroperoxybenzoic acid), heat (5 days at 80°C) and light (5 days at 1000 foot-candles). Only the base stressed sample did not undergo measurable degradation. In the remaining samples, recovery was between 36% and 94%, showing degradation did occur. These samples were evaluated by photodiode array detection and standard algorithms were used to evaluate the peak purity. In all cases, spectral analysis of the doretinel peak in the stressed samples shows the chromatographic system yields an analyte peak free from interference. The three-dimensional chromatogram for the worst case sample, acid hydrolysis, is shown in Figure 4. Degradation profiles for the other stress conditions did not contain any peaks not seen in the acid hydrolysis sample. Figure 5 contains multiple peak ratios for the acid hydrolysis sample, demonstrating that under these worst case stress conditions, the doretinel peak is free from interference.

TABLE I

Retention Time, Relative Retention Time, Capacity Factor and Resolution Data for Doretinel and Synthesis Related Compounds

Compound	Retention Time (minutes)	Relative Retention Time	Capacity Factor	Resolution
Benzylphosphonate[A]	2.1	0.21	1.4	28
Tetralone[B]	2.6	0.26	2.1	13
Acid Analog (II)	8.5	0.86	9.0	2.4
Hydroxy Analog (III)	8.8	0.89	9.4	2.0
Doretinel (I)	9.9	1.0	11	
Ester Analog (IV)	80	8.1	93	37

A = Benzoic acid, 4-[(diethoxyphosphinyl)methyl]-, ethyl ester
B = 7-Acetyl-3,4-dihydro-1,1,4,4-tetramethyl-2(1H)-naphthalenone

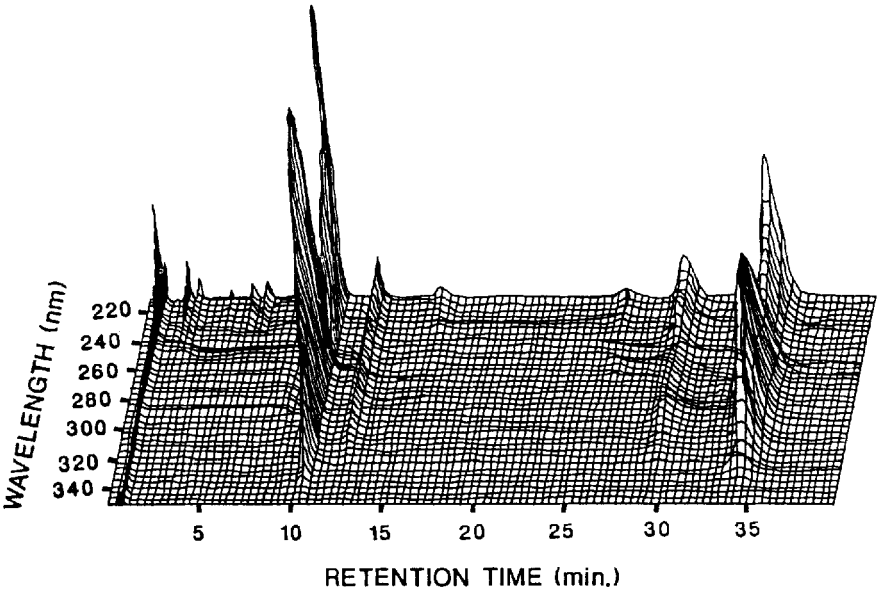


Figure 4: Three-dimensional chromatogram for acid stressed sample.

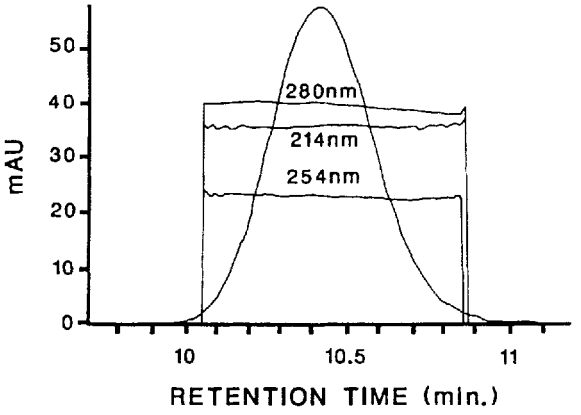


Figure 5: Wavelength ratios versus 300 nm for doretinel in the acid-stressed sample. Constant ratios over the entire peak are observed in the absence of co-eluting components.

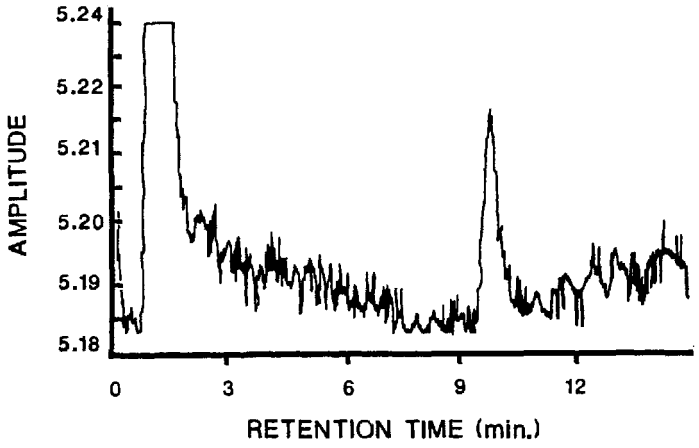


Figure 6: Chromatogram at the 2.4 ng detection limit.

Linearity and Detection Limit

Linearity and detection limit were determined by injecting doretinel samples ranging from 0.00478 to 0.956 μg/mL. At injection volumes of 500 μL, this corresponds to 2.4 to 478 ng on-column. The detection limit was determined to be 2.4 ng on-column, with a signal-to-noise ratio of approximately three. A chromatogram of doretinel at the detection limit is shown in Figure 6. Linearity is observed from 2.4 (the detection limit) to 191 ng on-column. Regression analysis of area vs. concentration data yielded a y-intercept of -612 (on a scale of 150,000) and a correlation coefficient greater than 0.999. The method is linear over nearly two orders of magnitude.

Precision

The precision of the chromatographic system was evaluated by determining the RSDs of areas and retention times for

TABLE II
Precision Data for Ten Consecutive Injections

Area Counts	Retention Time (minutes)
88903	9.59
90834	9.61
92002	9.93
89921	9.43
91560	9.67
89650	9.59
91290	9.65
91399	9.67
89526	9.42
91654	9.74
Average = 90674	Average = 9.63
%RSD = 1.19	%RSD = 1.51

10 consecutive injections of a 0.212 μg/mL doretinel standard solution. The data are summarized in Table II. The overall RSDs are 1.19% and 1.51% for area and retention time, respectively.

Recovery

Recovery data for the solid-phase extraction sample preparation scheme was generated by spiking placebo gel samples which had been placed in the barrel of a disposable syringe fitted with an extraction cartridge. Following preparation 500 μL aliquots were injected onto the HPLC system. The doretinel was detected at 300 nm. The levels examined were 0.7 to 10 ppm of doretinel. Table III lists the recoveries at these levels. Average recovery was 98.4%. Significance testing (alpha = 0.01, two-sided t-test) shows the average recovery is not statistically different from 100%. Linear regression

TABLE III
Recovery Data For Doretinel Gels by Solid-Phase Extraction

ppm Added	ppm Found	%Recovered
0.765	0.756	98.8
	0.737	96.3
	0.756	98.8
0.106	1.10	104
	0.991	93.5
	1.02	96.2
5.29	5.19	98.1
	5.11	96.6
	5.19	98.1
10.6	10.7	101
	10.5	99.1
	10.6	100
	Average =	98.4
	%RSD =	2.71

analysis of the recovery data yielded a slope of 1.00 (recovery equals 100%) and a y-intercept of -4×10^{-6} Significance testing (alpha = 0.01, two-sided t-test) shows the y-intercept is not statistically different from zero and the slope is not statistically different from one indicating that the method is accurate and unbiased.

In summary, a simple, rapid HPLC method has been developed and validated for release, uniformity and stability assays of the drug product. The method uses a sample clean-up step that takes advantage of the differences in solubilities between doretinel and a problematic excipient. Validation data show the method is precise, accurate, linear, unbiased and specific.

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