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Short communication

Development of an HPLC method for the analysis of Apomine in a topical cream formulation

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Abstract

A stability indicating, reversed-phase high performance liquid chromatographic method was developed for the quantification of Apomine, tetraisopropyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-ethyl-1, 1-bisphosphonate, in a topical cream formulation. Analysis of Apomine in the cream formulation was performed through a dilution of the cream base with tetrahydrofuran. This allowed the current method to bypass extraction and/or centrifugation for direct injection and analysis. Separation was achieved using an Alltima C_{18} 5 μm, 150 mm × 2.1 mm column and employed a gradient procedure, beginning with acetonitrile–water (65:35, v/v), at 0.6 mL/min for 9 min, followed by a rinse with isopropyl alcohol for 9 min. The complete gradient method has been optimized to separate Apomine from the nonpolar cream components, wash and equilibrate the column in a 30-min assay. This report demonstrates that this method is effective for quantification of Apomine in a cream formulation.

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Keywords: Apomine; RP-HPLC method; Cream formulation; Topical formulation

1. Introduction

According to the American Cancer Society, more then 1 million cases of skin cancer occur each year [1]. Apomine (Fig. 1), tetraisopropyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-ethyl-1, 1-bisphosphonate, is a novel antineoplastic agent, which is one of a family of compounds that inhibits the mevalonate/isoprenoid pathway of cholesterol synthesis [2–4]. Apomine accelerates the degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR). HMGR is an enzyme that catalyzes the first step of the mevalonate pathway, leading to the syntheses of cholesterol and other isoprenoid compounds. As a result of this mechanism, Apomine inhibits the growth of cancer cells from a number of cancer types, including lung, colon, breast and skin. Additionally, it induces apoptosis in tumor cell lines derived from leukemia, colon, liver, ovary and breast cancers [4]. The Arizona Cancer Center has conducted a Phase I clinical trial of orally administered Apomine, which indicated positive results for potential effects against ovarian cancer and melanoma [5]. Currently, Apomine is being developed for topical application as a potential chemopreventive agent for skin cancer.

Prior to a clinical study, preformulation studies are conducted in order to determine the stability of the raw drug substance and the drug in vehicle. To this end, a stability indicating reversed-phase high performance liquid chromatographic (RP-HPLC) method was developed and validated for analysis of Apomine both under aqueous conditions and in a topical formulation. Previously published methods for the analysis of an active pharmaceutical ingredient (API) in a cream formulation typically involve extraction with an organic solvent or some type of a centrifugation step, to separate the API from the excipients [6–14]. These procedures can increase both the amount of time required for sample preparation and/or the variability of the assay. The purpose of this work was to develop and validate an RP-HPLC method, employing UV detection, which would allow for analysis of the API without the need for an extraction or centrifugation step. The developed assay was evaluated for five consecutive days, in order to determine both the intra- and inter-day variation. The current method will be employed to

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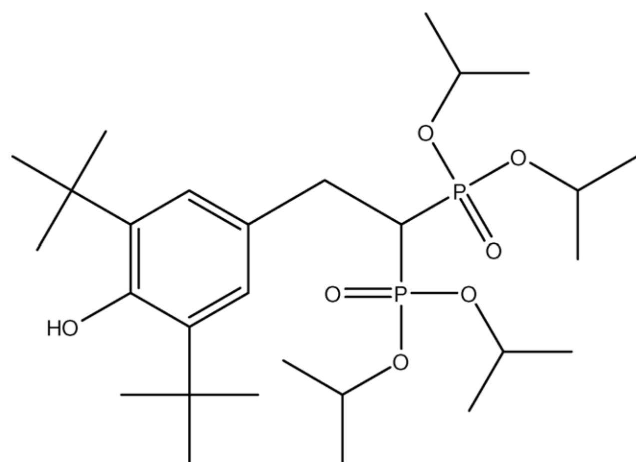

Fig. 1. Structure of Apomine.

test the stability of Apomine topical creams for use in clinical trials.

2. Experimental

2.1. Materials

Apomine was supplied by ILEX Oncology (San Antonio, TX, USA). Tetrahydrofuran (THF) and Isopropyl Alcohol (IPA), both of HPLC grade, were purchased from Burdick and Jackson (Muskegon, MI, USA). HPLC grade Acetonitrile (ACN) was purchased from EMD (Gibbstown, NJ, USA). A Millipore water purification system with a 0.22-μm filter was utilized for water. Apomine creams were compounded at 1% (w/w) in Dermabase cream (Paddock Laboratories Inc., Minneapolis, MN, USA).

2.2. Instrumentation

The HPLC system consisted of a Waters 2690 separation module (Waters, Milford, MA, USA) coupled with a Waters 996 Photodiode array (PDA) detector. An Alltima C_{18} 5 μm, 150 mm × 2.1 mm (Alltech Associates, Deerfield, IL) column, at 30 ± 2 °C, was used for the separations. The injection volume was 5 μL and the analytical wavelength was 210 nm. Instrument control, data acquisition, processing and peak symmetry were completed with the Millenium[32] Chromatography Manger Software (Waters, Milford, MA, USA).

A gradient chromatographic method was employed for the separation of the API from its degradation product and the cream excipients. Conditions of the method were as follows: ACN–H_2O (65:35, v/v) with a flow rate of 0.6 mL/min for the initial 9 min, followed by a 1-min linear gradient to IPA (100%) with a flow rate of 0.35 mL/min. Pure IPA was run at 0.35 mL/min for nine mins followed by a 4-min linear ramp to ACN–H_2O (65:35, v/v). Initial conditions of ACN–H_2O, at flow of 0.4 mL/min, were then run for 6 min, in order to equilibrate the column for the next injection. Total run time was 30 min.

Mass spectroscopy analyses (LC–MS) were performed on a Finnigan MAT TSQ 7000 (Waltham, MA, USA). A standard solution of Apomine was introduced into the mass spectrometer via a Hewlett Packard 1050 HPLC system (Palo Alto, CA, USA). Analyses of Apomine were conducted in Q1 MS mode with the following parameters: scan type-full; polarity-positive; mass range: 100–1200 *m/z*. Atmospheric pressure chemical ionization (APCI) source operating parameters: heated capillary temperature: 250 °C; vaporizer temperature: 400 °C; corona discharge current: 4.0 μA. Chromatographic separation was performed using the above described column with 20 min isocratic conditions of ACN–H_2O (65:35, v/v) with a flow rate of 0.6 mL/min.

2.3. System suitability

The HPLC system was equilibrated with the initial mobile phase composition, followed by six injections of the same standard. These six consecutive injections were used to evaluate the system suitability on each day of method validation.

2.4. Standard preparation

The primary stock solution of Apomine was prepared by analytically weighing approximately 25 mg of Apomine and diluting to a final volume of 50 mL with ACN–H_2O (65:35, v/v), resulting in a stock solution at or near 500 μg/mL. Serial dilutions were performed, with ACN–H_2O (65:35, v/v), to make six standards, ranging from 20 to 250 μg/mL. These standards were then stored at 4 °C and replaced every 2 weeks. Three standard calibration curves were injected periodically throughout the HPLC procedure, in order to establish linearity of detector response to analyte concentration.

2.5. Preparation of 1.0% cream (w/w) solution

Analytically weighed portions of approximately 0.2 g of 1.0% (w/w) Apomine cream were diluted with 10 mL of THF. Samples were sonicated (Bransonic-1510R, Bransonic Ultrasonics Corporation, Danbury, CT) for 10 min. Aliquots were then transferred to HPLC vials for injection.

3. Results and discussion

3.1. Method development

3.1.1. Development and optimization of Isocratic HPLC conditions

Initial assay parameters were based on physical-chemical properties of Apomine. Due to the high octanol–water partition coefficient (LogP), 6.09 [15] of the parent compound, a C_{18} column was selected for development. Based on the LogP and the melting point, 106 °C, Apomine has a calculated water solubility of 3.89×10^{-7} moles/l [16,17]. Therefore a reversed-phase assay was deemed most appropriate for initial testing. A UV scan of Apomine showed a maximal absorbance at or near 210 nm.

Initial method development was conducted on a C_{18} 5 μm, 150 mm × 2.1 mm column, operated at 30 ± 2 °C. This column provides efficient and reproducible separations of nonpolar compounds while minimizing solvent usage. Consequently, it was

selected for method development and remains the column utilized in the validated assay.

Preliminary method development of suitable isocratic conditions to resolve Apomine, on the C_{18} column, was conducted with ACN–H_2O as the mobile phase. A mobile phase of ACN–H_2O (65:35, v/v) was found to provide a reproducible, baseline resolved peak with an average peak tailing factor of 1.59. These conditions allowed for separation of Apomine ($t_r \sim 8.2$ min) from its major degradation product ($t_r \sim 6.2$ min).

3.1.2. Sample development

Previous work by Gupta and Myrdal [18], as well as Benjamin and Conley [19], suggested IPA and THF as potential solvents to explore as a means to dissolve the cream base and analyze Apomine without an extraction. Gupta and Myrdal had previously shown that IPA could be used to rinse nonpolar cream components from a C_{18} column; therefore initial solubility screening was conducted with IPA.

Various amounts of cream, in the range of 0.15–0.3 g, were sonicated for 20 min in 20 mL of IPA. Following sonication, the solutions were inspected with a Tyndall light to determine if the cream base was completely dissolved. Observations indicated that the cream base was not sufficiently soluble in IPA. Similar procedures were repeated with THF. It was found that ∼0.2 g of cream base were soluble in 20 mL of THF. The volume of THF was decreased to 10 mL while maintaining the same cream weight and the cream remained completely soluble. In the interest of decreased sample preparation time, sonication time was later reduced to 10 min. Inspection of the solution indicated that the cream base was completely dissolved in the THF, following the reduced sonication period.

3.1.3. Development and optimization of gradient HPLC conditions

Development of the gradient method for the separation of Apomine and the major degradation product from the cream excipients used the initial isocratic conditions as a foundation. From Section 3.1.2, it was known that the cream base was soluble in IPA at low concentration. Consequently, IPA was selected as the mobile phase component to elute the nonpolar cream excipients from the column. Method development began with a 45 min assay, consisting of a 12 min isocratic initial run followed by a linear ramp to IPA (100, v/v), to elute nonpolar cream excipients, followed by system equilibration to initial isocratic conditions. All flow rates, except initial isocratic conditions, were in the range of 0.15–0.2 mL/min. While these parameters proved effective for separation and acceptable peak symmetry of 1.56, a shorter run time was desired.

In order to decrease assay time, flow rate and time in each mobile phase composition were varied for all but the initial isocratic conditions. Based on the pressure limitations of the column and the HPLC system, flow rates were maximized to decrease assay time without adversely affecting the system or the column. Typical pressure throughout the gradient method ranges from 2750 to 3250 psi. The length of time in each portion of the assay was varied to accommodate a 30-min run time. Final mobile phase conditions are as described in Section 2.2. Example chromatographs of 1.0% cream and placebo are shown in Fig. 2A and B, respectively.

Due to the decreased run time and the absence of any extraction method, column carryover of cream excipients was a potential concern for subsequent analyses. To explore this, dissolved cream samples were injected, analyzed under optimized conditions, and followed by blank THF injections. The chro-

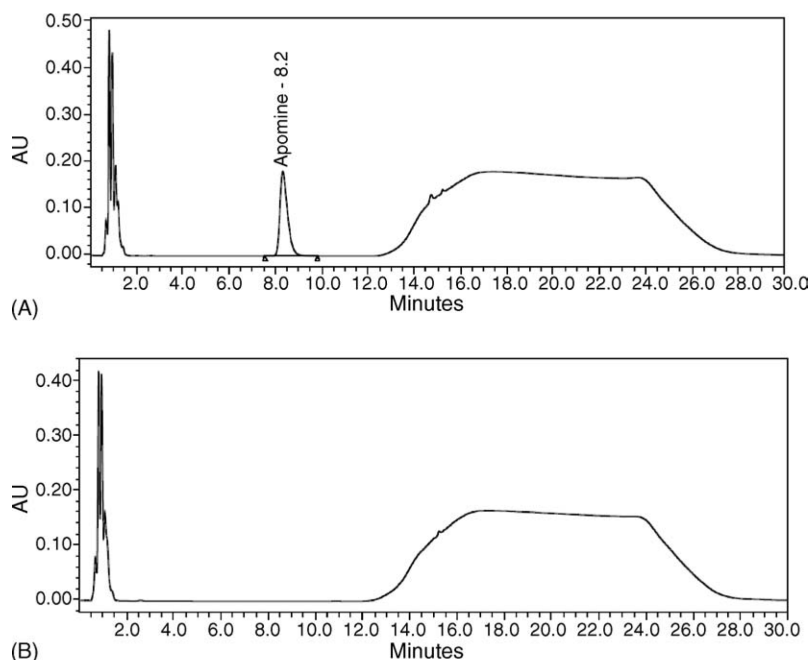

Fig. 2. Chromatographs of Apomine cream samples under isocratic conditions, 1.0% cream (A) and placebo cream (B).

matograms resulting from blank THF injections indicated that there was no carryover in the system.

3.1.4. Standard development

Initial mobile phase, ACN–H_2O (65:35, v/v), was utilized as the standard diluent for Apomine standards. Standard stability testing was conducted by comparison of aged samples with new standards. While stored at 4 °C, the AUC and slope of the calibration line determined by HPLC were not significantly different at 2 weeks. Standards were, therefore, determined to be stable for 2 weeks. Cream samples would not be dissolved in the same ACN–H_2O diluent; therefore, further testing of standard diluents was required. Standards were prepared as described in Section 2 utilizing THF as the diluent. The characteristics of the resultant standard curve were not significantly different from those of the ACN–H_2O standard curve characteristics. Therefore, it was determined that Apomine concentration could be analytically determined in THF.

3.2. Method validation

3.2.1. System suitability

In order to establish system suitability for the instrument, six consecutive injections of Apomine were made from one standard solution each day, prior to the validation injections, for each of the 5 days of method validation. The average concentration of the six injections, for each of 5 days, was evaluated both intra- and inter-day. These data are shown in Table 1, along with standard deviation (S.D.) and coefficient of variation (C.V.). Inter-day concentrations were analyzed by ANOVA and determined to not be significantly different ($p = 0.48$).

3.2.2. Linearity

To determine linearity, seven standard solutions of increasing concentration were injected in triplicate on each of the 5 days. The resultant calibration curve encompassed the expected concentration range of the 1% cream samples prepared per the Section 2 section above. The calibration curve was prepared by plotting the area under the response from the detector (AUC) line versus the concentration and analyzed through least squares regression (Table 2). The assay was found to be linear in the range from 20 to 500 μg/mL. Calibration curves were linear on all 5 days, with a slope of near 12,000 each day and an average correlation coefficient of ≥0.9995. The difference between the experimental and calculated concentrations is represented by the relative error (R.E.); the low absolute value of the R.E. shows the absence of scatter of the data from the linear best-fit line.

Table 1
System suitability, both intra- and inter-day, for an Apomine stock solution

System suitability					
Day	*N*	Average concentration		S.D.	C.V. (%)
1	6	139.28		0.7350	0.5277
2	6	138.98		0.4220	0.3036
3	6	138.85		0.1738	0.1252
4	6	138.92		0.2952	0.2125
5	6	138.91		0.3395	0.2444
Inter-day			Mean	138.99	
			S.D.	0.1685	
			C.V. (%)	0.1212	

Table 2
Linearity of HPLC method for analysis of Apomine cream

Linearity					
Day	*N*	Slope	S.D.	C.V. (%)	r^2
1	3	12170	15.10	0.1241	0.9995
2	3	12094	15.10	0.1249	0.9996
3	3	11857	16.07	0.1356	0.9995
4	3	11867	13.11	0.1105	0.9996
5	3	11939	21.46	0.1797	0.9996
	Mean	11985			
	S.D.	140.16			
	C.V. (%)	1.1695			

Sample ($N = 15$)	Concentration added (μg/mL)	Concentration recovered (μg/mL)[a]	S.D.	C.V. (%)	R.E.
1	22.6	24.4	1.24	5.0923	0.0817
2	45.2	46.6	0.16	0.3356	0.0302
3	90.4	92.6	0.42	0.4488	0.0239
4	135.6	138.6	0.38	0.2729	0.0219
5	180.8	182.9	0.43	0.2375	0.0115
6	271.2	274.0	0.48	0.1741	0.0102
7	452.0	448.0	0.70	0.1554	−0.0089

[a] Calculated from slope above.

3.2.3. Accuracy and precision

The accuracy of this method was determined as the percentage of the theoretical drug recovered from the 1.0% Apomine cream stored at both 4 and 25 °C. Mean drug recoveries for 4 and 25 °C were 99.37 and 100.86% with standard deviations of 1.03 and 0.86, respectively. Actual concentration recovered and the corresponding S.D., C.V. and R.E. are shown for each storage temperature in Table 3. These data convey that the assay is highly accurate based on the recovery of drug from the cream matrix.

For the purposes of testing the 1.0% Apomine cream, the method will be considered accurate and precise at a level that is ±5% of label claim. The recovery of Apomine from the cream was well within ±5% for each of the storage temperatures over the course of 5 days, thereby establishing that the method has the precision required.

3.2.4. Limit of quantitaition and detection

The limit of detection (LOD) and limit of quantitation (LOQ) of this method were determined from the standard deviation of the response of a known concentration of Apomine, as per ICH Q2B guidelines [20,21]. The LOD for this assay, calculated from three times the noise level of the response, is 1.56 μg/mL. The LOQ for this assay, calculated from ten times the noise level of the response, is 4.74 μg/mL.

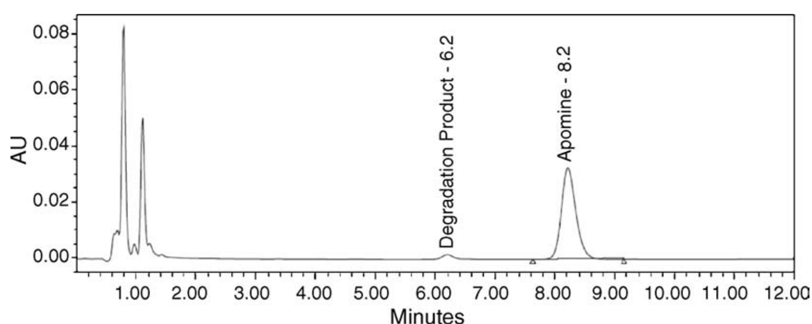

Fig. 3. Chromatogram of Apomine analysis indicating a resolved degradation product of Apomine (retention time = 6.2 min) analyzed under isocratic conditions.

Table 3
Accuracy and precision of HPLC analysis of Apomine cream (1.0%, w/w)

Day	*N*	Calculated cream concentration (%, w/w)	S.D.	C.V. (%)	R.E.
25 °C storage					
1	6	1.001	0.0034	0.3357	0.0059
2	6	1.010	0.0022	0.2141	0.0147
3	6	1.002	0.0017	0.1686	0.0074
4	6	1.007	0.0023	0.2319	0.0125
5	6	1.023	0.0089	0.8668	0.0278
	Mean	1.009			
	S.D.	0.0086			
	C.V. (%)	0.8563			
4 °C storage					
1	6	0.986	0.0038	0.3878	−0.0094
2	6	1.007	0.0037	0.3691	0.0117
3	6	0.990	0.0033	0.3362	−0.0051
4	6	1.002	0.0032	0.3198	0.0075
5	6	0.984	0.0051	0.5171	−0.0113
	Mean	0.994			
	S.D.	0.0103			
	C.V. (%)	1.0332			

3.2.5. *Specificity and selectivity*

The selectivity of the assay for Apomine is shown in the chromatographs in Fig. 2. The placebo shows no detector response near retention time of Apomine: 8.2 min, while the Apomine sample displays a peak which is baseline resolved from any interferences. The specificity of this method has been confirmed with both mass spectrometry and library spectra matching, using a photo diode array detector. LC–MS analyses resulted in a chromatograph with a single baseline resolved peak of similar retention time seen on the HPLC system. Concurrently, the MS indicated a molecular ion (*m*/*z* 563) which corresponds to the molecular weight, plus one, of Apomine ($m + H^{+}$). Further confirmation of peak identity was conducted using data collected with a 996 PDA detector and analyzed with Millenium[32] Chromatography Manger Software. A library spectrum was used to confirm peak identity and the absence of any impurity that possesses a different UV spectrum from Apomine. These results support the specificity and selectivity of this assay for Apomine.

3.2.6. *Degradation studies*

The initial isocratic conditions of the assay were utilized to conduct stability studies for Apomine under aqueous conditions (data not shown). Under these conditions, Apomine has one principle unidentified degradation product, with a retention time of 6.2 min, chromatograph shown in Fig. 3. A degraded sample of Apomine was, therefore, spiked into the dissolved cream base, for analysis via the validated assay. The unidentified degradation product again elutes at 6.2 min and is well resolved from the parent drug. This demonstrates the ability of the assay to concurrently monitor for this degradation product while determining Apomine content in the cream form.

4. Conclusions

An optimized RP-HPLC assay, employing UV detection, has been developed and validated for Apomine in a cream base. The gradient RP-HPLC assay allows for the separation of Apomine, degradation product and the cream components, without the need for an extraction procedure. The linearity, precision, accuracy, reproducibility, selectivity and stability-indicating nature of this assay have been established, and have been deemed sufficient to support clinical trials of topically applied Apomine creams.

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