JOURNAL OF
EMERGING INVESTIGATORS

Article

High-performance liquid chromatography insight in pH-dependent hydrolysis of andrographolide acetonide

Emily Shu[1,2], Madeleine Lloyd[1,3], Anushree Marimuthu[1,4], Anika Kulkarni[1,3], Rosie Chen[1,5], Srishti Venkatesan[1,6], Samyukta Athreya[1,7], Riya Kulkarni[1,7], Jonathan Wang[1,8], Aditya Sreedhar[1,9], Nadia Bianco[1,10], Kaitlyn Huynh[1,3], Eric Han[1,11], Adrian Gore[1,12], Raymond Chen[1], Akira Yamamoto[1], Edward Njoo[1]

[1] Department of Chemistry, Biochemistry & Physics, Aspiring Scholars Directed Research Program, Fremont, CA
[2] I rvington High School, Fremont, California
[3] Notre Dame High School, San Jose, California
[4] Mission San Jose High School, Fremont, California
[5] Adrian C. Wilcox High School, Santa Clara, California
[6] Leigh High School, San Jose, California
[7] American High School, Fremont, California
[8] Valley Christian High School, San Jose, California
[9] Fremont High School, Sunnyvale, California
[10] Washington High School, Fremont, California
[11] St. Francis High School, Mountain View, California
[12] Dougherty Valley High School, San Ramon, California

SUMMARY

Andrographolide is a natural labdane diterpenoid that functions as an inhibitor of Nuclear factor Kappa B (Nf-κB), and has demonstrated anti-inflammatory, anti-depressant, and anti-cancerous properties. In a one-step chemical synthesis, the 3,19-diol system of andrographolide can be protected with an acetonide group, yielding 3,19-isopropylidene andrographolide ("andrographolide acetonide"). Acetonide protecting groups are known to be unstable under mildly acidic conditions, and previous literature has reported that andrographolide acetonide is more potent than andrographolide. This led us to believe that 3,19-isopropylidene andrographolide exerts its biological activity as a pH-dependent prodrug of andrographolide. Using high performance liquid chromatography to create time courses that quantify the rate at which the acetonide group cleaves off of andrographolide acetonide in various mildly acidic pH buffers, we determined that the rate of hydrolysis was a first order reaction. In order to ascertain which mechanism of hydrolysis is more probable, we utilized density functional theory (DFT) to calculate the absolute free energies of the two initial rate-determining protonated intermediates, coming to the conclusion that C19 is preferentially protonated over C3 by 4.33 kcal/mol. Understanding the nature of andrographolide acetonide hydrolysis under different conditions lays the groundwork for improving both the stability and potency of andrographolide analogs via chemical synthesis.

INTRODUCTION

Natural products have been used in therapeutic applications since the beginning of human civilization. Such products and their analogs have more recently been the source of inspiration for the discovery and development of therapeutically relevant small molecules. One such compound is andrographolide, a labdane diterpenoid isolated from the medicinal plant *Andrographis paniculata.* Andrographolide has been used in Ayurvedic medicine but has recently garnered attention as a modern treatment for HIV/AIDS, multiple sclerosis, and various cancers through the inhibition of Nuclear Factor Kappa B (Nf-κB), a protein transcription factor involved in a myriad of cell signaling pathways (1-12).

Synthetic analogs of andrographolide have demonstrated more potent biological activity than their natural counterpart; one such analog is 3,19-isopropylidene andrographolide, hereafter referred to as andrographolide acetonide (13) **(Figure 1)**. The improved biological activity of andrographolide acetonide results from increased bioavailability, or the ability of a drug to be used by the body (14). Conveniently, this compound can be synthesized from andrographolide in a one-step high-yielding reaction and also can serve as a gateway to the chemical synthesis of other analogs (2). In fact, acetonides and other ketals are commonly used protecting groups in organic synthesis, which can conveniently be removed under mildly Brönsted or Lewis acidic conditions (14).

While acetonides are a convenient protecting group in organic chemistry, this raises the question of whether andrographolide acetonide is a prodrug, which has implications for its biological mode of action. A prodrug is a biologically inactive version of a drug that is metabolized into an active version after it enters the body. Installation of the acetonide protecting group turns the less potent andrographolide into andrographolide acetonide, a more potent congener. However, andrographolide acetonide could instead be functioning as

https://doi.org/10.59720/22-281

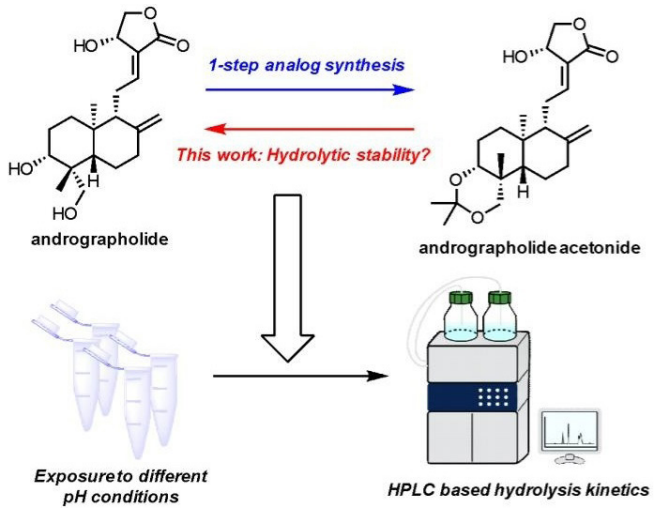

Figure 1: Workflow for the hydrolysis study of andrographolide acetonide. Diagram demonstrating scientific questions and procedural workflow to conduct HPLC analyses of acid catalyzed andrographolide acetonide hydrolysis. Procedure was initiated with the synthesis of andrographolide acetonide. Upon synthesis, hydrolytic stability of the compound in various pH conditions was tested using high performance liquid chromatography (HPLC) based hydrolysis kinetics, providing insight into the exact rate of andrographolide acetonide hydrolysis in accordance with environmental pH.

a prodrug of andrographolide. Andrographolide has been known to interact with lysosomes, which have a mildly acidic pH of around 4 (15). If andrographolide itself is the biologically active compound, andrographolide acetonide could be a simple prodrug of andrographolide which remains inactive until released under acidic conditions. Determining the mechanism behind the increased potency of andrographolide acetonide impacts future efforts in understanding the structure-activity relationship of andrographolide and its analogs.

Given the lability of acetonide groups under acidic conditions, we hypothesize that andrographolide acetonide would more readily hydrolyze under lower pH conditions as acetonide groups can be deprotected via acidic conditions. We performed qualitative experiments by thin-layer chromatography (TLC) to justify our hypothesis (13). However, TLC experiments are only qualitative, and we wanted to quantify the rate at which andrographolide acetonide hydrolyzes at different pH conditions. We set out to do this by using reversed-phase high-performance liquid chromatography (HPLC), an instrument-based analytical method that allows for the quantitative and real-time determination of the absolute concentration of analytes in a solution. To this end, we developed a reversed phase high performance liquid chromatography (RP-HPLC) method to quantitatively measure the rate at which acetonide hydrolysis occurs to convert andrographolide acetonide back to andrographolide **(Figure 1, 2)** under various conditions. By doing so, we were able to determine that, consistent with our hypothesis, andrographolide acetonide undergoes more rapid hydrolysis under lower pH conditions. Further, these

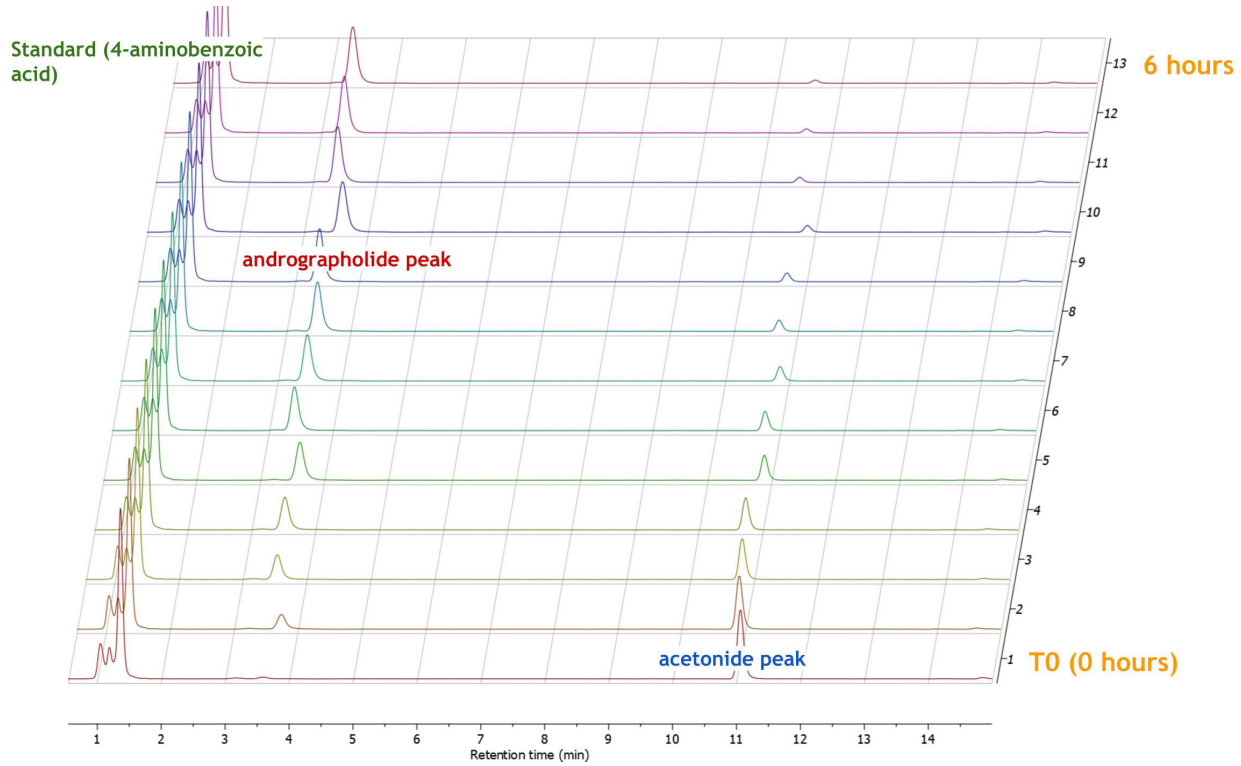

Figure 2: Time course assay of hydrolysis of andrographolide acetonide in pH 5 acetate buffer using high performance liquid chromatography (HPLC). Overlaid chromatograms of andrographolide acetonide hydrolysis into andrographolide in pH 5 acetate buffer conditions. Reaction completion is achieved in roughly 6 hours until andrographolide acetonide detection ceases, and injections are taken every 30 minutes. Numbers on the right correspond to the time of each injection.

https://doi.org/10.59720/22-281

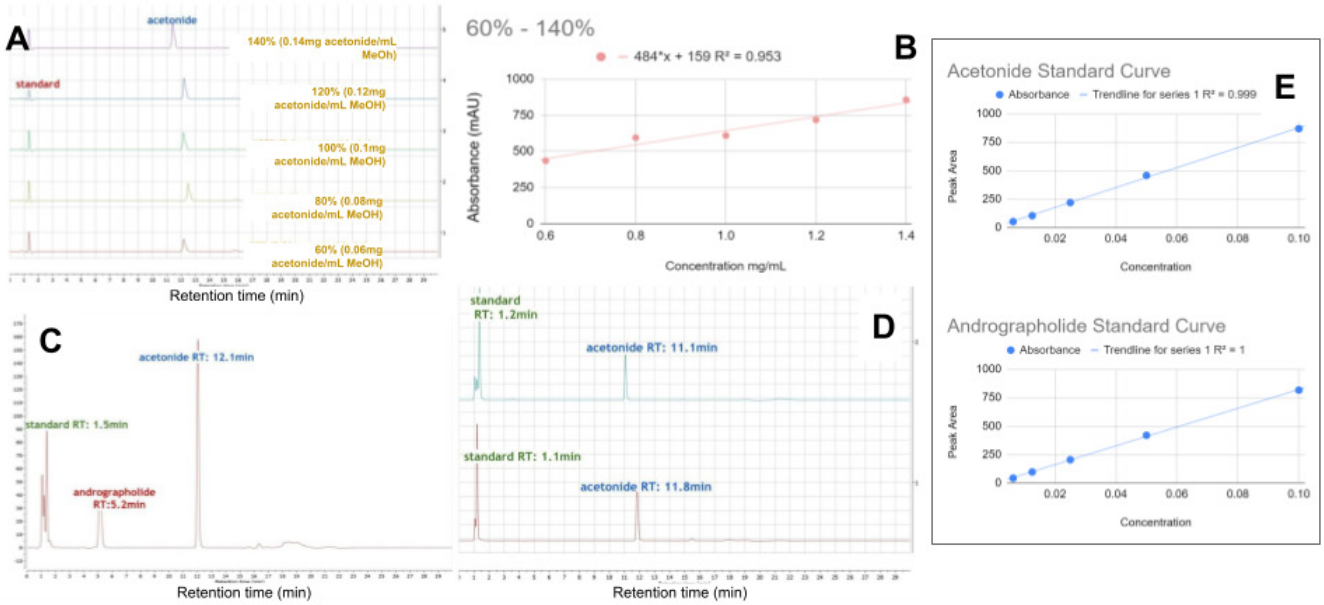

Figure 3: Method Validation (A) Linearity: overlaid chromatograms of andrographolide acetonide and standard (4-aminobenzoic acid) retention times. RP-HPLC, n = 5. (B) Linear regression of andrographolide acetonide standard curve, from 60% to 140% of test sample concentration. n = 3. Quantification of our experiments plotted absorbance against concentration using different standard curves. (C) Chromatogram of andrographolide acetonide-andrographolide separation with internal standard included. (D) Intermediate Precision: overlaid chromatograms of andrographolide acetonide and 4-aminobenzoic acid retention times. Two different tests were performed by two different analysts on two different days, at T0 in pH 5. n = 2. (E) Standard curves for andrographolide acetonide and andrographolide were used to interpret data when measuring the concentration of andrographolide and andrographolide acetonide over time.

experiments, along with computational modeling, suggest that the rate-determining step of andrographolide acetonide hydrolysis is initial protonation by a hydrogen ion, supporting our conclusion that andrographolide acetonide hydrolyzes more readily in more acidic conditions.

RESULTS

High Performance Liquid Chromatography

We used high performance liquid chromatography (HPLC) to test our hypothesis as it allows for the identification of both andrographolide and andrographolide acetonide in our sample. Furthermore, using HPLC allows for the quantification of the concentration of both compounds present in our sample by separating compounds and eluting them at different points in time based on their chemical properties. By measuring the relative concentration of both compounds in our reaction mixtures, we were able to determine the rate of hydrolysis from andrographolide acetonide into andrographolide.

A previously published method was optimized for our use as a result of premature hydrolysis of andrographolide acetonide and insufficient separation of the two compounds in our sample (16).

The developed HPLC method was validated according to parameters of linearity, specificity, sensitivity, and intermediate precision. Linearity was validated in accordance with International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, with five different samples between 60% to 140% of the sample concentration (17) **(Figure 3A)**. The linear regression equation was derived as y = 484x + 159 (R^2 = 0.953) **(Figure 3B)**, indicating a proportional relationship between the sample concentration and absorbance.

Specificity was assured via a consistent retention time of andrographolide acetonide **(Figure 3C)**, as well as effective separation between andrographolide acetonide and andrographolide. Separate standard solutions of the two compounds were also injected in order to verify consistent retention time. The sensitivity of the developed HPLC method was evaluated by testing different concentrations of the sample. The lowest and highest concentrations tested in the development of calibration curves were 6.25% and 1000% of the test concentration respectively, both of which were detected and quantified at consistent absorbance values. Intermediate precision was validated by comparing data from different analysts and assays from different days. Assays that were run by two different analysts on two different days were comparable **(Figure 3D)**.

pH-dependent Hydrolysis Assays of Andrographolide Acetonide

HPLC was used to develop an optimal method in order to quantify the rate of hydrolysis initially observed by thin layer chromatography. With an optimized method to separate andrographolide and andrographolide acetonide, analysis of the rate of hydrolysis indicates that andrographolide acetonide hydrolyzes at a faster rate in lower pHs and follows first-order reaction kinetics **(Figure 4, 5)**.

Upon plotting the concentration of the remaining starting material over time, an exponential trendline returned the best correlation; likewise, the plot of the natural logarithm of the concentration of andrographolide acetonide over time gave the best linear fit **(Figure 5)**. Based on these results, the reaction rate of andrographolide acetonide hydrolysis with respect to the concentration of andrographolide acetonide

https://doi.org/10.59720/22-281

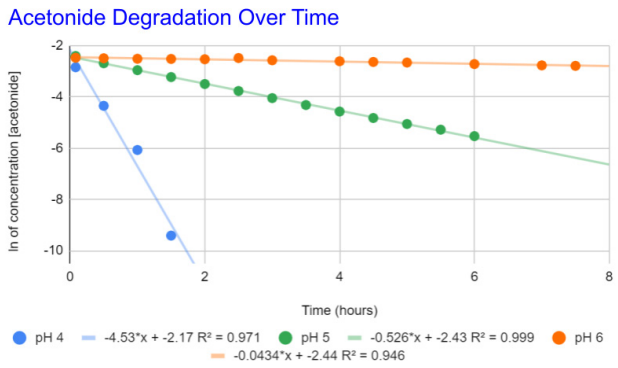

Figure 4: Natural log of the concentration of andrographolide acetonide was used to determine the observed rate constant and reaction kinetics of andrographolide acetonide hydrolysis. Overlaid linear regression of andrographolide acetonide hydrolysis over time in pH 4, 5, and 6 acetate buffers, via linear regression of natural log of concentration of andrographolide acetonide over time. RP-HPLC was performed with an Agilent 1100 and Waters 2695 HPLC instrument, with each hydrolysis run repeated 3 times for consistency.

was empirically determined to be first order. The observed hydrolysis rate constant for pH 4, 5, and 6 between three runs averaged to k = 4.53 1/s (R^2 = 0.971), k = 0.526 1/s (R^2 = 0.999), and k = 0.0478 1/s (R^2 = 0.977), for each pH respectively. Knowing the reaction proceeded as a first order reaction with respect to only andrographolide acetonide concentration, further investigation involved determining the key rate-determining step of hydrolysis.

Consistent with our expectations, andrographolide acetonide was found to hydrolyze at a faster rate at lower pHs. Specifically, the rate of hydrolysis scaled linearly with the concentration of H^+ evidenced by an approximately ten-fold increases in hydrolysis rate per lowered pH unit **(Figure 4)**. Thus, we postulate that initial protonation of the ketal by a proton is the rate-determining step for andrographolide acetonide's mechanism of hydrolysis **(Figure 5)**.

Rationalizing the Mechanism of Hydrolysis Using Density Functional Theory

Hydrolysis of the acetonide could proceed either through initial protonation at the C3 oxygen or C19 oxygen (**Figure 5**). Following this, the six-membered ring opens into a resonance-stabilized dimethyl oxocarbenium. This highly reactive species is attacked by water to continue the hydrolysis process. Through proton transfer and other addition and elimination events, acetone is lost as a byproduct; at the end of this process, andrographolide is liberated.

In order to rationalize which of these pathways may be operative, we performed density functional theory (DFT) free energy calculations to determine the absolute free energy of these highly reactive protonated states at the B3YLP, def2-SVP levels of theory. We used DFT over other wet-lab techniques because wet-lab techniques would not be able to distinguish between the two possible mechanisms of hydrolysis, as opposed to DFT. Through DFT, we found that C19 is preferentially protonated over C3 by 4.33 kcal/mol, determined by DFT calculations from the single point energies of optimized conformers of both protonated states.

DISCUSSION

Andrographolide and its analogs present important therapeutic leads in several biomedical indications, especially in anti-cancer activity, anti-viral activity, and others (1-7, 10-11, 14-15). However, quantitatively understanding the hydrolytic stability of these compounds is essential for understanding their *in vivo* mode of action in any possible metabolic liabilities. Through our study, we hoped to quantify the rate at which andrographolide acetonide would hydrolyze back into andrographolide in different pH buffers as a preliminary method of determining whether or not andrographolide acetonide is a prodrug. We utilized reverse-phase high performance liquid chromatography (RP-HPLC) to develop a quantitative method to determine the rate of hydrolyis of andrographolide acetonide back into andrographolide.

Consistent with our mechanistic model of the hydrolysis of andrographolide acetonide into andrographolide **(Figure 5)**, the rate of hydrolysis was observed to be first order correlating with both andrographolide acetonide and H^+ concentrations. As the pH condition of the buffer increased by 1, the reaction constant (k), calculated via the natural log of concentration over time, decreased by approximately ten-fold, starting at k = 4.53 1/s at pH 4 and ending at k = 0.0478 1/s at pH 6, with the coefficient of determination (R2) being 0.971 and 0.977 respectively for each line of best fit. The rate determining step of initial protonation supports our hypothesis that andrographolide acetonide will more readily hydrolyze under acidic conditions.

We noted that the actual absorbance of andrographolide and acetonide slightly differs from the value we used for method development. Despite this limitation, our conclusion remains unchanged as our calculations for absorbance of each compound were determined by a standard curve of each compound. While a different buffer system may have been better suited for a pH level of 6, we found that other buffers tested were incompatible with either the assay or intrinsic product stability. Thus, acetate buffer was chosen as it had the most appropriate buffer range. Furthermore, the internal standard 4-aminobenzoic acid was not qualitatively analyzed due to substandard spectra obtained, as it was added to confirm appropriate retention time and not quantify the concentration of our analytes. Other internal standards that were not tested but may be more optimal include trimethoxybenzene, though we hypothesized that the different protonation states of the internal standard resulted in its three signals.

Our findings touch on the potential of andrographolide acetonide functioning as a prodrug, and this could be true of other andrographolide analogs. While here we have investigated the hydrolysis of the acetonide ketal protecting group on andrographolide acetonide, a similar mechanism of hydrolysis occurs in the hydrolysis of acetals on andrographolide. Studies investigating the structure-activity relationship of hydrolytic stability of these compounds are currently underway; moreover, *in vitro* hydrolysis studies to determine whether or not andrographolide acetonide is a prodrug in various cell media environments with acidic conditions are currently a subject of investigation in our laboratory. As a whole, this work demonstrates a significant conclusion in understanding the mode by which andrographolide acetonide is more potent than andrographolide, supporting the broader hypothesis that in

https://doi.org/10.59720/22-281

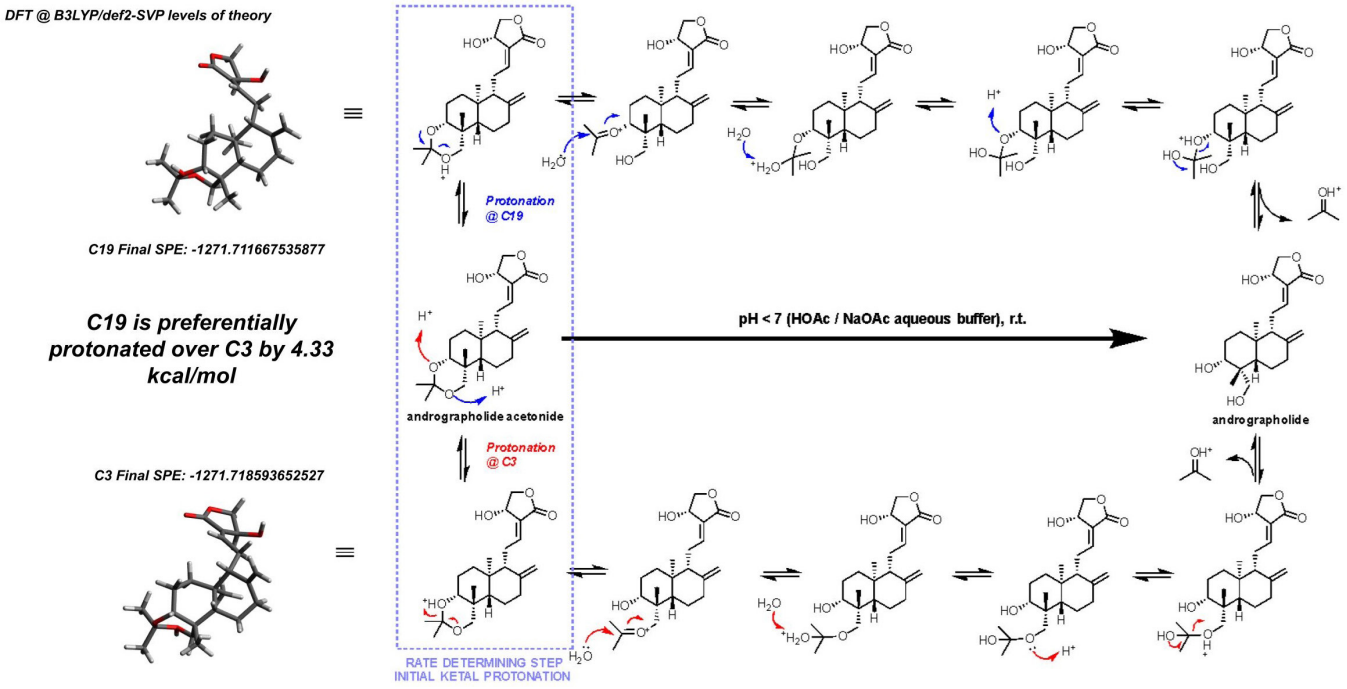

Figure 5: Elucidating the mechanism of hydrolysis based on the stability of the highly reactive protonated intermediates using Density Functional Theory (DFT). Diagram depicting the reaction mechanism of andrographolide acetonide hydrolysis into andrographolide. Possible rate determining steps include initial protonation at C3 oxygen or C19 oxygen. Density functional theory (DFT) free energy calculations were performed to determine the absolute free energy of the protonated states at the B3YLP, def2-SVP levels of theory.

mildly acidic conditions, andrographolide acetonide acts as a prodrug rather than a more potent analog.

MATERIALS AND METHODS

Chemical Synthesis

The andrographolide acetonide was synthesized in our laboratory using a previously reported protocol (12).

Computer Modeling

Compounds were constructed virtually on Avogadro and optimized using DFT on ORCA, an open source quantum chemistry software package. Following this, DFT-optimized structures had single point energy calculations performed on a Dell Poweredge 710 server with a 24 core Intel Xeon X5660 processor @ 2.80GHz and 32GB RAM. All DFT calculations were modeled with the dielectric constant of water acting as a solvation model, B3LYP was the basis set, and def2-SVP was the functional.

Preparation of Working Standard Solutions

Acetic acid (Sierra Chemical) and sodium acetate (Sigma-Aldrich) were utilized for the creation of acetate buffers. Sodium hydroxide (Belle Chemical LLC) and hydrochloric acid (VWR International LLC) were utilized for pH adjustment.

High performance Liquid Chromatography: Sample Preparation and Method Development

0.1% trifluoroacetic acid (TFA) (AK Scientific) was incorporated within solvent systems in the original methods tested. 4-aminobenzoic acid (Sigma Aldrich), was used as an internal standard in samples.

Method development, validation, and hydrolysis assays were performed on two HPLC systems: an Agilent 1100 and Waters Alliance 2695, both utilizing deuterium lamps and quaternary pumps, as our laboratory is equipped with multiple HPLC instruments. Data acquisition and analysis were performed on ChemStation and Empower Pro, respectively.

We first began by using a previously reported method for quantifying andrographolide concentration, but there was inadequate separation between andrographolide and andrographolide acetonide in our chromatograms (18-19). We then sought to improve the separation by omitting the use of trifluoroacetic acid (TFA) in our mobile phase **(Figure 6)** and changing the gradient from 0 to 100% methanol to 30 to 100% acetonitrile in 15 minutes instead.

In initial method development, multiple sample injection volumes, from 1.0 µL to 10 µL, were tested; and unusual pressure fluctuation at some sample injection volumes led to the conclusion that 2.0 µL was the optimal injection volume.

Initially, the UV spectrum of andrographolide and andrographolide acetonide demonstrated adequate absorbance at 230 nm via spectrophotometry (Thermo Fisher Nanodrop 1000 UVVis); maximal absorbance was later observed at 245 nm, which was the spectroscopic range utilized in the final method. Methanol was originally used as a second mobile phase, but was replaced with acetonitrile as a result of background noise in the chromatogram.

To improve peak resolution, 0.1% trifluoroacetic acid (TFA) was initially included as a mobile phase additive but was found to result in the premature degradation of the analyte prior to UV detection **(Figure 6a)**. Proper separation between andrographolide acetonide and andrographolide peaks was achieved with the exclusion of TFA, and the solvent gradient was edited to achieve better separation of the two compounds

https://doi.org/10.59720/22-281

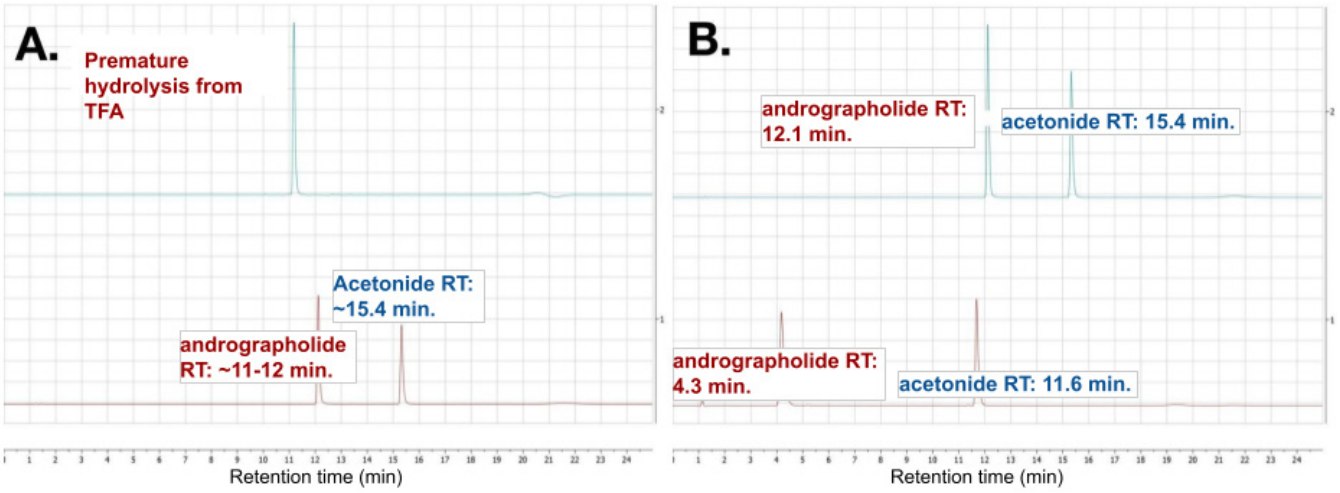

Figure 6:Method development of solvent systems and gradients. Overlaid chromatograms of andrographolide-andrographolide acetonide separation for method development. (A) Shown on the top, the presence of trifluoroacetic acid (TFA) caused premature hydrolysis of andrographolide acetonide which could not be quantitatively measured. Thus, it was removed from the solvent system, resulting in visible separation as shown in the bottom chromatogram. (B) Adjustment of the mobile phase gradient to achieve greater separation between the two compounds. At the top, the gradient was from 0% to 100% methanol in 15 minutes. As shown on the bottom chromatogram, this was adjusted to 30% to 100% acetonitrile in 15 minutes. Greater separation with a difference of about 7 minutes was accomplished by adjusting the original method, which had a separation difference of about 3 minutes in comparison.

(Table 1, Figure 6b).

The method used in both the Waters 2695 and Agilent 1100 HPLC systems were standardized for all variables, excluding pressure and temperature. The pressure range was set to 0.0 to 4000 psi on the Waters 2695, and 0 to 5801.51 psi on the Agilent 1100, with runs immediately aborting if the pressure was outside of the range on both machines. C18, 2.1x100mm 3 micron columns were used (ThermoFisher Hypersil) on both machines.

Chromatography

Acetate buffers were used to track andrographolide acetonide hydrolysis at pH 4, 5, and 6. We prepared 200 mL of 0.1 M acetate buffers for pH 4 and 5. A pH 6 buffer, stretching beyond the natural pH range of acetate buffers, was prepared using the recipe for pH 5 acetate buffer and was adjusted to pH 6 with 0.5 M sodium hydroxide.

For buffers, various quantities of acetic acid and sodium acetate were dissolved in 160 mL of deionized water to produce a 0.1 M solution, at different pHs. Additional deionized water was added to increase buffer volume to 200 mL. The pH of the buffer was confirmed using a pH meter.

Preparation of Working Standard Solutions

The stock standard solution of andrographolide was prepared in 100% methanol at a 1 mg/mL concentration, with fresh solutions created each day. Five working standard solutions with concentrations of 0.1, 0.05, 0.025, 0.0125, and 0.00625 mg/mL were prepared via serial dilutions of the stock solution in methanol for both andrographolide acetonide and andrographolide, to determine the relationship between absorbance and concentration for the two compounds. While new stock solutions were created each day, standard samples were run for the sake of consistency. As andrographolide and andrographolide acetonide remain stable when dissolved in methanol, the ten standard curve samples were run after the andrographolide acetonide samples in the HPLC carousel.

High performance Liquid Chromatography: Sample Preparation

For an internal standard, 4-aminobenzoic acid was used because it did not interfere with the reacting sample components and had a peak isolated from those of the sample. At a concentration of 0.17 mg/mL (1.0 µL/mL), 1.7 mg 4-aminobenzoic acid was dissolved in 100 µL of 0.1 M acetate buffer (pH 4-6); 10 µL of the 100 µL solution was pipetted into 890 µL of the acetate buffer to achieve the final concentration. Five minutes prior to the assay, 1 mg/mL solution of a andrographolide acetonide/methanol was dissolved in the buffer solution.

Sodium phosphate buffers were created from sodium phosphate dibasic heptahydrate (Sigma Aldrich) and sodium phosphate monobasic monohydrate (Sigma Aldrich) to monitor andrographolide acetonide hydrolysis in pH 6. However, an initial test with phosphate buffer produced poor trace quality. Citric acid buffers at pH 4, 5, and 6 with citric acid (Milliard) and sodium citrate (Spectrum Quality Products Inc.) were also tested and yielded cleaner chromatograms; however, citric acid was observed to slowly degrade over time. In order to avoid these issues, the buffer system was changed to solely an acetate buffer system, under which both

Initial Method				Finalized Method			
Minutes	Flow Rate (mL/min)	(100% H_2O)	(100% MeOH)	Minutes	Flow Rate (mL/min)	(100% H_2O)	(100% ACN)
0	0.3	100.0	0.0	0	0.25	70.0	30.0
15	0.3	0.0	100.0	15	0.25	0.0	100.0
16	0.3	100.0	0.0	16	0.25	70.0	30.0
25	0.3	100.0	0.0	25	0.25	70.0	30.0

Table 1: Initial and Final HPLC Solvent Gradient. Initial gradient consisted of methanol (MeOH) and water, with methanol progressing to 100% within 15 minutes. Finalized method consisted of acetonitrile (ACN) and water, with acetonitrile progressing from 30% to 100% within 15 minutes.

https://doi.org/10.59720/22-281

of these challenges were circumvented. As the natural pH of acetate buffers ranges from pH 3.6 to 5.6, 0.5 M sodium hydroxide was utilized in the production of pH 6 acetate buffer to raise the pH.

ACKNOWLEDGMENTS

We would like to thank Aspiring Scholars Directed Research Program for providing us a space to perform our research and the Olive Children Foundation for funding our research. We would also like to thank our lab technicians for ensuring our safety throughout the entire process.

Received: December 2, 2022
Accepted: October 23, 2024
Published: May 1, 2025

REFERENCES

1. Calabrese, Carlo, et al. "A Phase I Trial of Andrographolide in HIV Positive Patients and Normal Volunteers." *Phytotherapy Research*, vol. 14, no. 5, 28 July 2000, pp. 333-338. https://doi.org/10.1002/1099-1573(200008)14:5<333::AID-PTR584>3.0.CO;2-D.
2. Uttekar, Mayur M., et al. "Anti-HIV Activity of Semisynthetic Derivatives of Andrographolide and Computational Study of HIV-1 gp120 Protein Binding." *European Journal of Medicinal Chemistry*, vol. 56, Oct. 2012, pp. 368-374. https://doi.org/10.1016/j.ejmech.2012.07.030.
3. Ciampi, Ethel, et al. "Efficacy of Andrographolide in Not Active Progressive Multiple Sclerosis: a Prospective Exploratory Double-Blind, Parallel-Group, Randomized, Placebo-Controlled Trial." *BMC Neurology*, vol. 20, no. 1, 7 May 2020, p. 173. https://doi.org/10.1186/s12883-020-01745-w.
4. Bertoglio, JC, et al. "*Andrographis paniculata* Decreases Fatigue in Patients with Relapsing-Remitting Multiple Sclerosis: a 12-Month Double-Blind Placebo-controlled Pilot Study." BMC Neurology, vol. 16, no. 77, 23 May 2016. https://doi.org/10.1186/s12883-016-0595-2.
5. Lim, Jonathan Chee Woei, et al. "Andrographolide and its Analogues: Versatile Bioactive Molecules for Combating Inflammation and Cancer." *Clinical and Experimental Pharmacology and Physiology*, vol. 39, no. 3, 21 Oct. 2011, pp. 300-310. https://doi.org/10.1111/j.1440-1681.2011.05633.x.
6. Reabroi, Somrudee, et al. "The Anti-cancer Activity of an Andrographolide Analogue Functions Through a GSK-3β-independent Wnt/β-catenin Signaling Pathway in Colorectal Cancer Cells." *Scientific Reports*, vol. 8, no. 7924, 21 May 2018. https://doi.org/10.1038/s41598-018-26278-8.
7. Reabroi, Somrudee, et al. "A Silyl Andrographolide Analogue Suppresses Wnt/β-catenin Signaling Pathway in Colon Cancer." *Biomedicine & Pharmacotherapy*, vol. 101, May 2018, pp. 414-421. https://doi.org/10.1016/j.biopha.2018.02.119.
8. Alugoju, Phaneidra, et al. "Chapter 7 - Phytotherapy for Breast Cancer." *A Theranostic and Precision Medicine Approach for Female-Specific Cancers*, 6 Nov. 2020, pp. 129-163. https://doi.org/10.1016/B978-0-12-822009-2.00007-8.
9. Malla, Rama Rao, et al. "Chapter 7 - Terpenoids as Potential Targeted Therapeutics of Pancreatic Cancer: Current Advances and Future Directions." *Breaking Tolerance to Pancreatic Cancer Unresponsiveness to Chemotherapy*, 26 Feb. 2019, pp. 111-116. https://doi.org/10.1016/B978-0-12-817661-0.00007-X.
10. Islam, Muhammad Torequl, et al. "Andrographolide, a Diterpene Lactone from *Andrographis paniculata* and its Therapeutic Promises in Cancer." *Cancer Letters*, vol. 420, 28 Apr. 2018, pp. 129-145. https://doi.org/10.1016/j.canlet.2018.01.074.
11. Mishra, Siddhartha Kumar, et al. "Andrographolide and Analogues in Cancer Prevention." *Frontiers in Bioscience-Elite,* vol. 7, no. 2, 1 Jan. 2015. https://doi.org/10.2741/E732.
12. Zhou, et al. "Reactivity-informed Design, Semi-synthesis, and Michael Addition Kinetics of C-ring Diversified Andrographolide Analogs." *Journal of Emerging Investigators*, Nov. 2022. https://emerginginvestigators.org/articles/21-258.
13. Greene, Theodora W., et al. "Protective Groups in Organic Synthesis." 3rd ed., *Wiley Interscience*, 1999, pp. 207-215, 716-719
14. Kongyingyoes, Bunkerd, et al. "3,19-isopropylidene Andrographolide Suppresses Early Gene Expression of Drug-resistant and Wild Type Herpes Simplex Viruses." *Antiviral Research*, vol. 132, Aug. 2016, pp. 281-286. https://doi.org/10.1016/j.antiviral.2016.07.012.
15. Zhou, Jing, et al. "Andrographolide Sensitives Cisplatin-induced Apoptosis via Suppression of Autophagosome-lysosome Fusion in Human Cancer Cells." *Autophagy*, vol. 8, no. 3, 2012, pp. 338-349. https://doi.org/10.4161/auto.18721.
16. Sajeeb, B, et al. "Identification and Quantification of Andrographolide from Andrographis paniculata (Burm. f.) Wall. ex Nees by RP-HPLC Method and Standardization of its Market Preparations." *Dhaka University Journal of Pharmaceutical Sciences*, vol. 14, no. 1, Jun. 2015. https://doi.org/10.3329/dujps.v14i1.23738.
17. "Validation of Analytical Procedures: Text and Methodology Q2(R1)". *International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use*, Nov. 2005. database.ich.org/sites/default/files/ Q2%28R1%29%20Guideline.pdf.
18. Patil, Rashmi and Jain, Vandana. "Andrographolide: A Review of Analytical Methods." *Journal of Chromatographic Science*, vol. 59, no. 2, 21 Nov. 2020, pp. 191-203. https://doi.org/10.1093/chromsci/bmaa091.
19. Syukri, Yandi, et al. "Validation of a Simple HPLC-UV Method For the Quantification of Andrographolide in Self-Nano Emulsifying Drug Delivery System (Snedds) For Dissolution Study." *International Journal of Drug Delivery Technology*, vol. 7, no. 4, Dec. 2017, pp. 22-26. https://doi.org/10.25258/ijddt.v7i1.8913.