AN ABSTRACT OF THE THESIS OF

Janelle Thimmesch for the Master of Science

in Forensic Science presented on

March 30, 2017

Title:

Identification and Quantification of Ibuprofen in Blood Using HPLC-UV/Vis.

Thesis Chair: Dr. Melissa Bailey

Abstract approved:_____

Non-steroidal anti-inflammatory drugs are some of the most frequently used medications in the United States. Ibuprofen is a common over-the-counter nonnarcotic analgesic, and it is also typically used as an antipyretic and as an anti-inflammatory. Intentional or unintentional ibuprofen overdose is common but typically found to be non-life threatening. If a victim had decreased hepatic or renal function, however, ibuprofen overdoses may impart significant toxicity, and at high dosages, it has been linked to cardiovascular events. Currently, the Sedgwick County Regional Forensic Science Center (RFSC) sends postmortem samples to a contract laboratory if ibuprofen toxicity is suspected. The purpose of this project is to develop and validate a method for the detection and quantitation of ibuprofen for RFSC using High Performance Liquid Chromatography (HPLC) with a UV/Vis detector. Ibuprofen and an internal standard, o-toluic acid were added to negative blood and extracted using acetate buffer (pH 4.5) and ethyl acetate/ hexane 50/50 over a range of concentrations (40 mg/L – 450 mg/L). Using the Breeze 2 software package, a calibration curve was generated and various test concentrations were quantitated. The method was validated in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines. Actual concentrations (as measured by the Breeze 2 software) were within ±20% of the expected concentrations. This method is a cost-effective option for rapid ibuprofen analysis.

Keywords: NSAIDs, Ibuprofen, Quantitation, HPLC, Validation

Identification and Quantification of Ibuprofen in Blood Using HPLC-UV/Vis.

A Thesis

Presented to

The Master of Science in Forensic Science Program

The Departments of Biological Sciences and Physical Sciences

EMPORIA STATE UNIVERSITY

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Janelle Catherine Thimmesch

May 2017

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Approved by the Department Chair

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Committee Member

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Committee Member

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Committee Member

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Dean of the Graduate School and Distance Education

ACKNOWLEDGMENTS

My deepest thanks to my thesis chair, Dr. Melissa Bailey, and committee members, Dr. Timothy Rohrig and Dr. Carlos Peroza. Their help in the writing of this thesis and assistance with the research will always be greatly appreciated. I would also like to express my thanks to Tyson Baird and Sam Miller for their assistance in the development of this research.

STYLE MANNUAL

This thesis was written according to the guidelines of the Journal of Analytical Toxicology.

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Introduction

Ibuprofen (IBU), figure 1, is a common over the counter (OTC) drug and is the third most highly consumed pharmaceutical in the world (Marchlewicz et al., 2017). Because OTC drugs are more easily accessible and are often viewed as less dangerous than prescription drugs, there is a greater chance of accidental overdose. It is considered part of the family of nonsteroidal anti-inflammatory drugs (NSAIDs) and part of the subcategory of propionic acid. In 1986 an estimated 100 million prescriptions were written for NSAIDs (Howard & Delafontaine, 2004). This figure does not account for the amount purchased OTC without a prescription. IBU is a nonnarcotic analgesic and is typically used for the treatment of pain. It is also an anti-inflammatory and an anti-pyretic medication. IBU is commonly over-dosed; however, the effects are typically not life threatening (Kunsman and Rohrig, 1993). The recommended dose of IBU can change depending on the severity of the pain with the average daily dose 900-2400 mg (Baselt, 2000).

Within the body, IBU is well absorbed after oral ingestion. Its half-life is 2 hours and reaches peak plasma concentration in 0.5-1.5 hours (Kunsman and Rohrig, 1993). The volume of distribution for the drug is 0.14 L/Kg (Baselt, 2000). The pKa of IBU is 4.91 (Ibuprofen). IBU is highly bound to plasma proteins, and as a result, has a potential for drug-drug interactions, including interaction with acetaminophen whose effects are increased in the presence of IBU. Studies have found that after ingestion of a 200 mg dose, a peak plasma concentration of 0.26 mg/L, was found after 1.5 hours. A 400 mg dose produces an average plasma concentration of 28 mg/L in 1.0-1.3 hours. The plasma

concentration after an 800 mg dose was 49 mg/L at the one hour mark (Baselt, 2000). In the presence of aspirin, IBU can increase the risk of cardiovascular disease by 75% (Howard & Delafontaine, 2004). The route of phase I metabolism for the drug is hydroxylation and carboxylation (Levine, 2015). CYP2C8, -2C9 and glucuronosyltransferase enzymes facilitate metabolism of IBU. The metabolism occurs within the liver (Klaassen, 2008). IBU is heavily metabolized by oxidation of the isobutyl group (Baselt, 2000). The LD_{50} in humans is unknown; however, in rats IBU has an LD_{50} of 636 mg/kg when administered orally (Toxnet, 2005).

IBU inhibits the enzymes cyclooxygenase-1 and -2 (COX-1, -2) (Howard & Delafontaine, 2004). The COX enzymes catalyze the conversion of arachidonic acid to various eicosanoids including the prostanoids prostaglandins (PGs) and thromboxanes. These prostanoids act on several tissues within the body including smooth muscle in various organs, kidney, and bone. More specifically, PGs help regulate inflammation, pain, and fever (Silverthorn, 2013). PGs are typically produced at sites of damage and regulate inflammation, blood flow, the formation of blood clots, and labor. Within the gastrointestinal (GI) tract, the decrease in PGs can lead to irritation as they maintain the GI mucosal barrier (Toxnet, 2005). COX-1 maintains the normal physiological state in the tissues within the kidney, GI tract, and platelets. COX-2 is induced by inflammatory stimuli including cytokines, endotoxins, and growth factors. Both enzymes are involved in cardiovascular homeostasis.

IBU blocks the access of arachidonic acid to the COX isozymes. This causes a decrease in the synthesis of PGs, thus leading to a decrease in pain and inflammation. As

IBU inhibits both COX, long-term use can lead to adverse side effects in the GI tract and increase the risk of cardiovascular events (Silverthorn, 2013). IBU is commonly used in the long-term treatment of chronic pain and inflammation diseases such as rheumatoid arthritis (Norman & Henry, 2012).

The inhibition of COX-1 has been linked to gastrointestinal problems including ulcers and bleeding due to chronic use of NSAIDs (Norman & Henry, 2012). To avoid the GI side effects, COX-2 inhibitors have been specifically developed, but it has been discovered that inhibition of the COX-2 enzyme leads to an increased risk of cardiovascular episodes and strokes. The specific COX-2 inhibitors increase the risk by promoting thrombosis (Howard & Delafontaine, 2004). Non-specific NSAIDs, such as IBU, will inhibit both COX-1 and COX-2 enzyme activation. Therefore, chronic use of IBU, exceeding 1200 mg/day, can lead to both gastrointestinal problems as well as cardiovascular risks (Mollersen et al.). Similarly, NSAIDs can cause acute renal failure (Klaassen, 2008). IBU inhibits renal PGs, which will decrease renal function when the kidney is PG dependent. This will decrease the glomerular filtration rate and the effective renal plasma flow within the kidneys. The renal system becomes PG dependent when the patient has hypovolemia, sodium depletion, chronic heart failure, and chronic renal failure. Consistent exercise in combination with NSAIDs also lead to a PG-dependent renal state and renal failure in athletes (Farquhar, 1999).

Previously, Sedgwick County Regional Forensic Science Center (RFSC) had no method for testing or quantifying for IBU in post mortem samples. As a result, all necessary testing for IBU was outsourced. The method developed in this project allows

for identifying and quantifying IBU within a blood sample. This method will be used by the RFSC to identify and quantitate IBU from post-mortem samples. In cases which require multiple analyses and in child death cases, and where sample size is limited, a test that requires a small volume is quite beneficial. The method uses 0.5 mL of the blood. The small amount of sample used allows more tests to be performed from the samples collected. The new method tests for the target analyte using a High Performance Liquid Chromatography (HPLC) with a UV/Vis detector. The identification of IBU is based upon its retention time relative to an internal standard. The method development and validation procedure follows the guidelines set by SWGTOX as described in "Standard Practices for Method Validation in Forensic Toxicology."

Experimental

The starting method for the research was based upon the Virginia Department of Forensic Science Standard Operating Procedure (VA SOP). The method was modified to include IBU as an analyte, in addition to acetaminophen and salicylic acid. All of the prepared reagents and stock solutions used complied with RFSC's Toxicology Standard Operating Procedure (Tox SOP).

All prepared stock solutions were made at a 1mg/mL concentration from solids for the internal standard and target analyte in reagent grade methanol. The concentration of the stock solutions was verified using the UV/Vis and the purity was verified using a Gas Chromatography Mass Spectrometer (GCMS). Stock solutions were prepared for o-toluic acid, figure 2, and IBU. From the stock solution, a 0.5 mg/mL working of solution IBU was prepared. Initially, stock solutions of acetaminophen (APAP) and salicylic acid (SA) were also prepared for inclusion in the method. To verify the purity of each standard, the stock solutions were tested on an Agilent GC System with a Network Mass Selective Detector. Stock solutions with impurities of less than 10% were accepted. The concentration of the solution was determined using an Agilent Uv/Vis. The solution was considered acceptable if the concentration was within 0.10 of the expected value.

The reagents were also prepared according to the Tox SOP. The extraction solvents were 0.1M Acetate buffer pH 4.5 and 50:50 Ethyl Acetate: Hexane. The acetate buffer was prepared by dissolving 2.93 g sodium acetate (trihydrate) in approximately 400 ml of nanopure deionized (DI) water, then 1.6 mL glacial acetic acid was added and the solution was brought to volume (q.s) to 500 mL with DI water. The pH was

measured using a pH meter to test for 4.5 ± 0.1, if required, with 0.1M sodium acetate or 0.1M acetic acid was added to adjust the pH appropriately. A 50:50 ethyl acetate: hexane mixture was prepared at a total volume of 1000 mL. The mobile phase A was a solution of nanopure DI H_20 with 1.5% acetic acid and mobile phase B is 100% optima grade Acetonitrile (ACN). A complete list of all reagents used is in Table 1.

Positive controls for the validation of the method were prepared in advance. The positive controls were prepared from a second stock solution. 600 µL, 1000 µL, and 3500 µL of the IBU stock solution (1 mg/mL) were individually transferred to 10 mL volumetric flasks. The samples were dried down to approximately half volume and DI H_2O was used to dilute to the original aliquot volume. Then the controls were q.s. to the one liter volume with negative defibrinated blood. The final concentration for each of the positive controls was 60 mg/L for the low, 100 mg/L for the medium, and 350 mg/L for the high standards. All of the controls were kept in the refrigerator when not in use. After the completion of the validation procedure, only the medium concentration control was used when performing the method with case samples.

Apparatus

The instrument used for analysis was a Waters HPLC with an in-line degasser and a UV/Vis detector. The column installed on the instrument was a Syncronis C-8 150 mm x 4.6 mm x 5 µm. The detector was set to 230 nm. The flow pressure of the system was set to 1700 psi -1950 psi, and pressure should hold stable with fluctuation ±1.2% of the total psi. The flow rate of the mobile phase was 2.0 mL/min with a beginning gradient flow rate of mobile phase A: 1.80 mL/min and mobile phase B: 0.20 mL/min, full description of flow rate method in Table 2. The method was set to run for 21.5 minutes per injection including a 2.5 minute hold in between each injection. Instrument parameters are listed in Table 1.

Methods

A calibration was prepared using five standards ranging from 40-450 mg/L. Each standards' concentration and aliquot volumes of the IBU working solution are listed in Table 3. The aliquots were added to screw cap test tubes and dried down under air at approximately 40˚C. After reaching dryness, 0.5 mL of defibrinated sheep's blood was added to the calibrator standards. O.5 mL of the defibrinated blood was also added to a clean tube to act as a negative control. 0.5 mL of each positive control were also aliquoted out in triplicate.

To each tube, 50 µL of the o-toluic acid internal standard stock solution was added, for a final concentration of 100 mg/L of o-toluic acid. Then all of the samples were vortexed. Next 1 mL of 0.1M acetate buffer pH 4.5 and 3 mL of 50:50 hexane: ethyl acetate were added. All of the tubes were capped and rotated for 30 minutes, then centrifuged for 15 minutes at 2500 rpm. The top organic layers were then transferred to new screw cap tubes using pasture pipettes. Finally, all of the samples were dried down under air at approximately 40˚C and reconstituted in 200 µL of 50:50 nanopure DI H_2O: Acetonitrile. All of the samples were stored in the refrigerator overnight at 4-8ºC

Data Collection and Analysis

The eluate was measured at 230 nm using a Uv/Vis detector and generated a chromatogram of the absorbance unit (AU) vs. time. The Waters' Breeze 2 software was used to control the instrument parameters and to view the chromatograms. The software was set up to generate the calibration curve from the standards and to quantitate the concentration of the analyte in relation to the internal standard. The peaks for each analyte were identified based upon their relative retention time and quantitation based upon the peak height.

Method Validation

A validation plan was generated to outline the criteria necessary according to SWGTOX and RFSC. To fulfill the requirements of the validation plan, the method needed to be performed five times, with three calibrators tested in triplicate. At least one of the runs had to be performed by a different analyist and different runs had to occur on different days to determine the robustness of the method.

The method was validated according to the following parameters. The correlation coefficient of the calibration curve had to be 0.9875 or greater. The three positive controls used were to determine the accuracy of the calibration curve for quantitation. The measurements were said to be acceptable if they were within 20% of the target concentration. The carry-over from the highest concentration had to be below the limit of detection (LOD). The LOD is the lowest concentration where the signal to noise ratio is less than three and the limit of quantitation (LOQ) is the lowest point on the calibration curve. Interference studies were performed with other common drugs of abuse and other available NSAIDs. The stability of the method was tested by performing the analysis on one set of standards a second time 72 hours later. All experimental concentration values were determined to be accurate if they were within 20% of the expected concentration.

In total, ten validation runs were performed. The method was performed for validation until five runs fit within the validation parameters described previously. The results of these runs are listed in Table 4. For the validation of this method, a toxicologist from the RFSC performed one of the validation runs, 6. The %CV was under 7% for the

study, indicating that the procedure is robust. A separate run was used to determine interference.

Linearity was assessed over a range of concentrations for the analyte. To be acceptable under the SWGTox guidelines, the correlation coefficient for the calibration curve had to be consistently greater than or equal to 0.9875. For IBU, the concentration range that provided the best correlation was 40 mg/L to 450 mg/L. The fit for the calibration curve is second order quadratic. For the five accepted validation runs, the data demonstrate an acceptable quadratic range and correlation consistently greater than 0.9875. Figure 3 shows the calibration curve for the final validation run performed. The correlation coefficient values for each run are listed in Table 4 and average at 0.9963.

The LOD could not be determined, as the standard deviation of the baseline could not be calculated. Due to the quadratic nature of the calibration fit, a linear model cannot be applied for estimating the LOD. As a result, the LOD was set equal to the lowest point on the calibration curve, 40 mg/L. Below this value, the potential IBU peaks could not be distinguished from baseline noise. The LOQ is the same as the lower limit of linearity, 40 mg/L. Figure 4 is a chromatogram of IBU at the LOD concentration. The LOQ is lowest point on the calibration curve and is equal to 40 mg/L. A lower concentration could not be obtained while maintaining the correlation value.

To determine potential carry-over in the method, negative samples were injected after the two highest points in the calibration curve. The analyte was only detectable in amounts under the LOQ. Only one negative control had a concentration of IBU above the LOQ, the negative following the highest calibrator in validation 3. A second known

negative was run and showed carry over under the LOQ. The chromatogram of the first negative control is Figure 5. The peak of IBU was quantitated at 158 mg/L. The second negative control performed was unable to be properly quantitated as the value was significantly below the LOQ. This suggests that even with high carry-over, the concentration of the next injection should be minimally affected.

Inter-day precision was determined for the target analyte with five runs over the course of as many days. Per run, each of the selected concentrations, low 60 mg/L, medium 100 mg/L, and high 360 mg/L, was tested in triplicate. This gave an n=15 for IBU at the low and medium concentrations and an n=14 at the highest concentration; the third high concentration injection in validation run 8 was outside of the accepted CV ≤ 20%. Table 4 contains the results from all of the controls tested, and Table 5 contains the calculated results of the precision of the method. The average concentration of the controls was 61±3 mg/L for the low, 95±5 mg/L for the medium, and 326±15 mg/L for the high. Figure 6 compares the expected concentration of the controls vs. the actual concentration. While the values differ from the expected, they are still within the ±20% acceptable deviation as stated in SWGTox. The calculated CV and Bias show that the measured concentrations are within 7% of the expected values of the controls.

The test for stability was set up to determine if standards and samples run on Friday could be run again on Monday without any loss in concentration. For this test, only the high and low controls were examined. Validation run 6 was run at the zero hour and was run again after 72 hours. A direct comparison was made of the concentration of IBU for all of the standards and the low and high controls (Figure 7). The difference in

concentration of each injection was calculated. This value was converted into a percentage of the original concentration and summated. The calculated difference between the concentrations of the two runs was -0.972%. Based upon this value, the prepared samples are stable up to 72 hours with refrigeration and can be used for analysis.

An interference study was conducted using additional NSAIDs: acetaminophen, naproxen, salicylic acid, and other common drugs of abuse, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), diphenhydramine, methadone, hydrocodone, zolpidem, verapamil, and trazodone (Figures 8-10). Important to note on figure 10, no peaks present are a result of the common drugs of abuse and the peak around 8.6 minutes is carry-over from the naproxen run performed before it. It was determined that these analytes did not have a negative impact on the analysis for IBU. The analytes showed no interference with the retention times or absorbance of IBU and the internal standard.

Discussion

For greatest efficiency within a crime lab, a testing method of this sort would include multiple NSAIDS. When this research was started, APAP and SA were included. The method developed was set to include them, but both were excluded over the course of the research. Figure 8 is a chromatogram of APAP, SA, IBU, and the internal standard extracted from whole blood. As shown in figure 8, a peak from the blood elutes out shortly before SA. This additional peak interfered with the identification and quantitation of the SA peak. The gradient method of the flow rate was altered to increase the resolution between the two peaks, but the peaks would not resolve. During some of the injections performed, their elution order switched, and in another injection, the peaks combined. Due to time constraints, SA was excluded from the method before the validation process was started.

APAP was initially included in validation study. It was the first drug to elute out around 2.5 minutes, shortly after the void volume. Noise was present along the baseline following the void volume. The Uv/Vis detection line was flushed in an attempt to decrease the noise, but it had no effect. The noise along the baseline made identifying low concentrations of APAP difficult and made the quantitation of the drug uncertain. Following the parameters set for the validation study, APAP excluded from the method for casework. With more time, APAP and SA could be included in the method.

Additional trouble shooting was performed to develop the best instrument method for the analysis. In the beginning, additional peaks were eluting and appearing on the chromatogram. Several methanol runs were performed to wash out the contamination,

and the additional "ghost" peaks continued to elute. Runs were performed where no injection was made and only the mobile phase was run. As no sample was injected, it was concluded that the injection port was not responsible for the contamination when the "ghost" peaks persisted. The syringes used for injection were taken apart and cleaned, but this did not remove the contamination peak. Therefore, it was concluded that the sample loop was contaminated with crystalized analyte from previous exams performed on the HPLC prior to the instruments storage. The sample loop was replaced and the "ghost" peaks no longer appeared on the chromatograms.

Another problem encountered during the method development was a large interference peak that appeared at random intervals during a series of runs (Figure 12). The peak typically eluted at the same retention time and interfered with the analysis of SA and the internal standard. A wash of the organic mobile phase was included at the end of each run to attempt to remove the interference. 1.5% of acetic acid was added to the organic mobile phase. This was done in an attempt to remove the blob and smooth the baseline. Figure 13 shows a baseline run chromatogram with the addition of 1.5% acetic acid to the organic mobile phase. The modified mobile phase did not improve the quality of the chromatograms. The column on the HPLC was replaced with a new C-8 column and the blob shape disappeared. The acetic acid was removed from the organic mobile phase as it decreased the ability to quantitate IBU because of the shape of the baseline.

Conclusion

This is a simple robust method chromatographic method for the detection and quantitation of IBU in whole blood. IBU can be quantitated over a range of 40-450 mg/L and maintain a correlation value of 0.9875. The precision of the method has a CV of less than 7%. The method also have good sample stability over 72 hours.

Further research for this method would include potentially changing the mobile phase. Acetic acid used in the aqueous mobile phase as a maximum wavelength detection at 230 nm; a different acid could allow for testing at different wavelengths. Setting up a gradient method for the detection wavelength, would optimize the method for the individual analytes. Potentially increasing the rate of the mobile phase gradient would allow for a shorter run time.

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Appendix

Figure 1: Structure of ibuprofen (Ibuprofen)

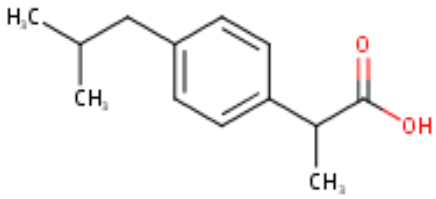


Figure 2: Structure of o-toluic acid. (o-Toluic Acid)

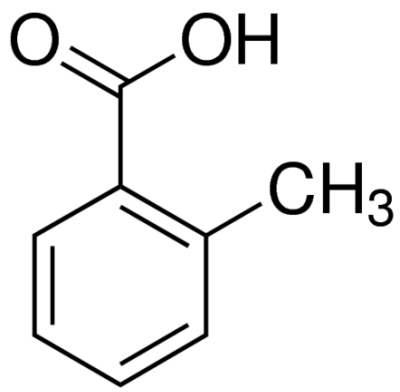


Table 1: A list of reagents and instruments used for the developed method.

Reagents	
• Reagent Grade Methanol • Reagent Grade Acetic Acid • Optima Grade Acetonitrile	• Sodium Acetate Buffer pH 4.5 • 50:50 Ethyl Acetate: Hexane
Standards & Controls	

• Internal Standard
  ○ O-Toluic Acid (1mg/mL)
• Target Analyte
  ○ Ibuprofen (1mg/mL)
	• Negative Control
	  ○ Defibrinated Sheep's Blood
	• Positive Controls
	  ○ Defibrinated Sheep's Blood and IBU

Instruments	
• Waters 1525 Binary HPLC Pump with Waters 2489 UV/Vis Detector and Waters In-Line Degasser AF operating Waters Breeze 2 software	• Agilent 6892 GC System with Agilent 5973 Network Mass Selective Detector • Agilent 8453 Uv/Vis

Table 2: The gradient method for mobile phase A and B concentration and the degree of the curve.

Time (min)	% A	% B	Curve
0.00	90	10	N/A
15.00	10	90	6
15.10	0	100	6
17.00	0	100	6
17.10	90	10	6
19.00	90	10	6

Table 3: The ibuprofen aliquot volumes and final concentrations for each standard tube of the calibration curve.

Standard (Tube)	Aliquot of stock solution (µL)	Final conc. of IBU (mg/L)
5	450	450
4	300	300
3	160	160
2	80	80
1	40	40

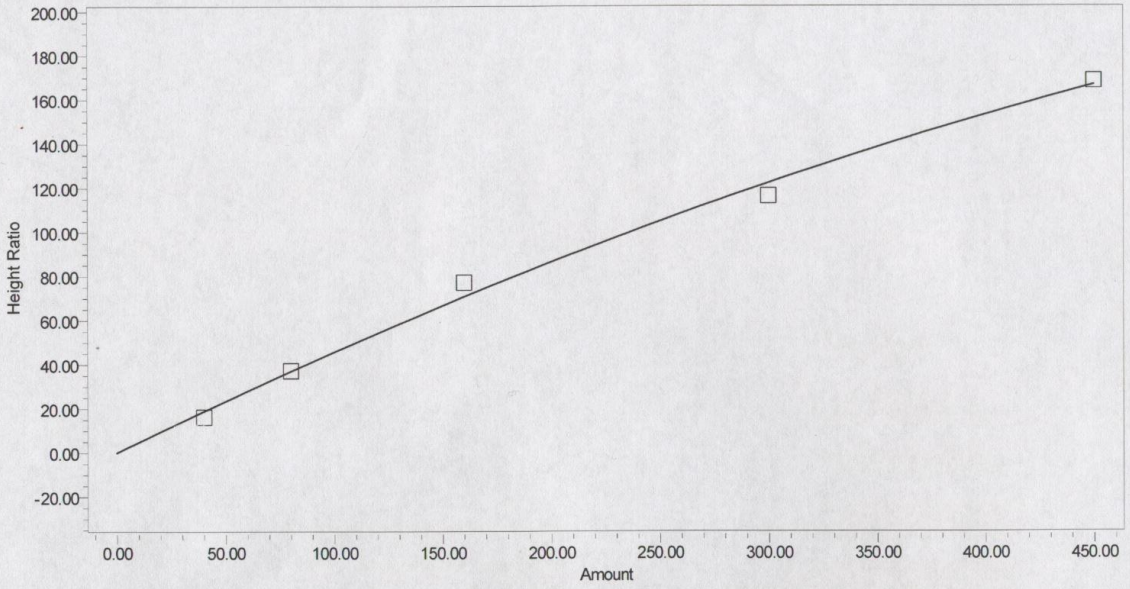
Table 4: Concentration results from accepted validation runs obtained from the Breeze 2 software and the correlation coefficients for each calibration curve.

	IBU (mg/L)		IBU (mg/L)		IBU (mg/L)
Low Expected	60.0	Medium Expected	100.0	High Expected	350.0
Validation 3 – R^2 = 0.997724					
Low 1	55.126	Med. 1	103.231	High 1	327.893
Low 2	61.143	Med. 2	93.56	High 2	315.214
Low 3	65.465	Med. 3	90.839	High 3	328.940
Average	60.578		95.877		324.016
Validation 4 – R^2 =0.996011					
Low 1	64.453	Med. 1	92.631	High 1	345.995
Low 2	56.137	Med. 2	90.472	High 2	337.871
Low 3	57.858	Med. 3	94.291	High 3	340.084
Average	59.483		92.465		341.317
Validation 6 – R^2 = 0.994427					
Low 1	65.234	Med. 1	81.755	High 1	328.958
Low 2	63.435	Med. 2	95.709	High 2	326.546
Low 3	60.367	Med. 3	98.266	High 3	359.962
Average	63.012		91.910		338.489
Validation 8 – R^2 = 0.998916					
Low 1	61.78	Med. 1	98.343	High 1	313.675
Low 2	64.545	Med. 2	98.103	High 2	310.039
Low 3	60.110	Med. 3	100.088	High 3	261.414
Average	62.145		98.845		295.043
Validation 9 – R^2 = 0.994251					
Low 1	60.205	Med. 1	99.888	High 1	324.163
Low 2	64.904	Med. 2	97.244	High 2	318.667
Low 3	54.983	Med. 3	90.733	High 3	294.538
Average	60.031		95.955		312.456

Table 5: Precision and accuracy calculations of the validation study for all of the positive controls.

	IBU (mg/L)		IBU (mg/L)		IBU (mg/L)
Low Expected	60.0 n=15	Medium Expected	100.0 n=15	High Expected	350.0 n=14
Mean =	61.049		95.101		326.610
SD =	3.56		5.126		15.762
%CV =	5.831		5.395		4.825
Bias =	1.748		4.989		6.682

Figure 3: Ibuprofen calibration curve from validation run 9.



Name: Ibu; Time: 10.810; Fit Type: Quadratic (2nd Order); R: 0.997122; R^2: 0.994251; Equation: Y = -2.45e-004 X^2 + 4.80e-001 X + 1.06e-001; Date Calibrated: 8/9/2016 2:50:12 PM CDT

Peak: Ibu

	Sample Name	Peak Name	Level	Amount	Response	Calc. Amount	% Deviation	Manual Point	Ignore Point
1	std1	Ibu		40.000	1.626e+001	34.233528	-14.416	No	No
2	std2	Ibu		80.000	3.724e+001	80.629414	0.787	No	No
3	std3	Ibu		160.000	7.712e+001	176.185805	10.116	No	No
4	std4	Ibu		300.000	1.163e+002	282.659149	-5.780	No	No
5	std5	Ibu		450.000	1.684e+002	456.973952	1.550	No	No

Figure 4: Chromatogram of ibuprofen at 40 mg/L demonstrating LOD.

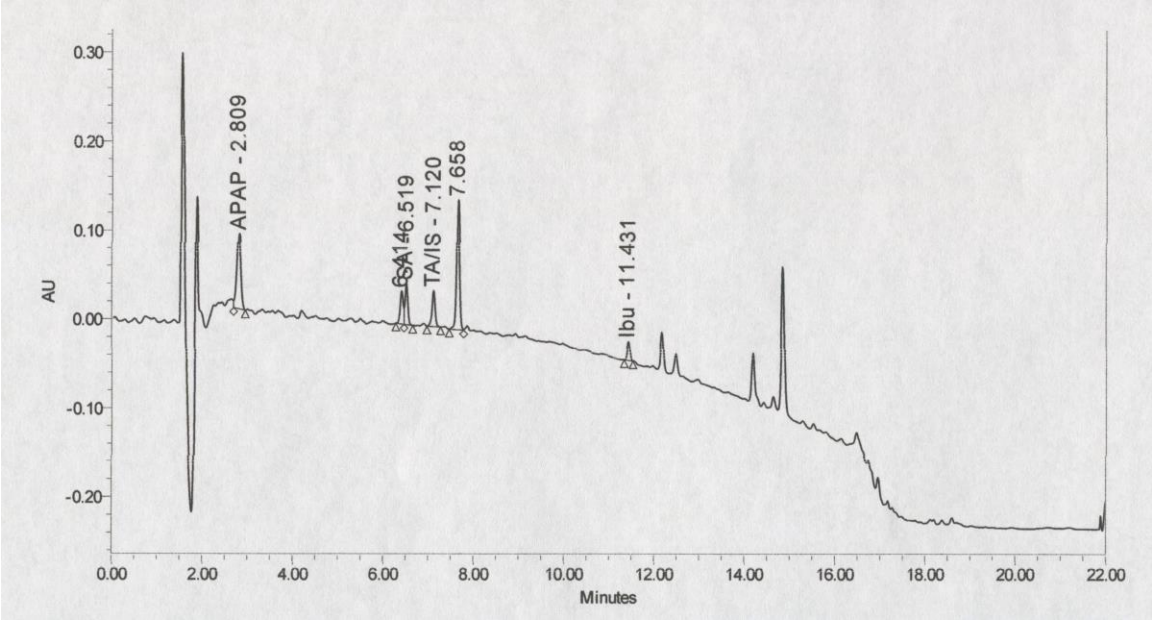


Figure 5: A chromatogram of a second negative control following a high concentration standard, demonstrated the carry-over below LOD.

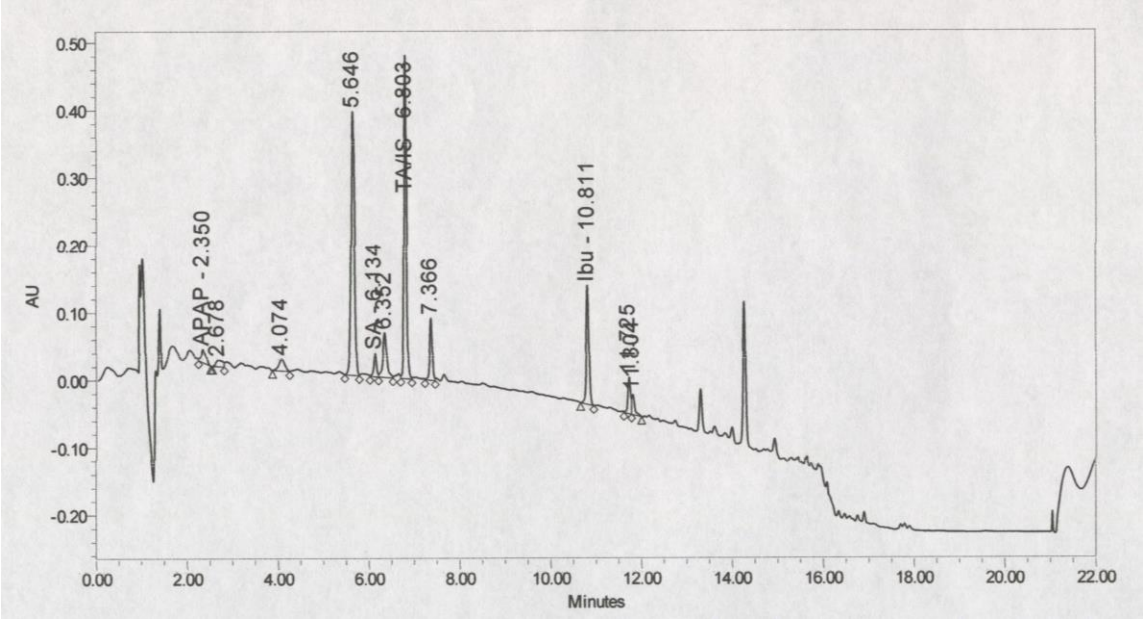


Figure 6: The average concentration of the positive controls compared to the expected concentration.

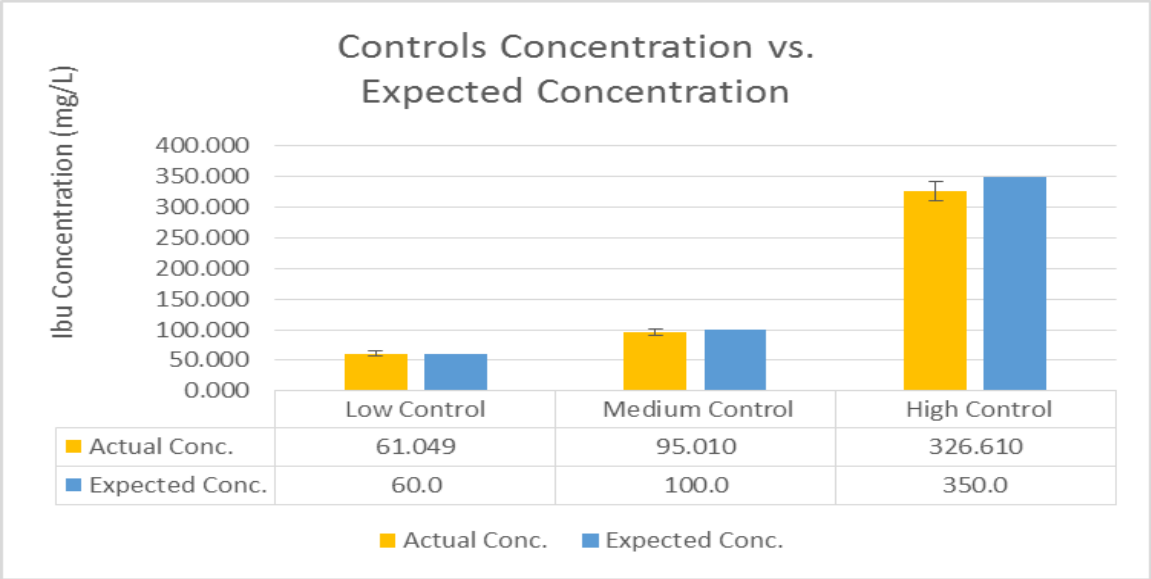


Figure 7: Validation run 6, time zero, compared to the second run, time 72 hours. Demonstrating stability of the method.

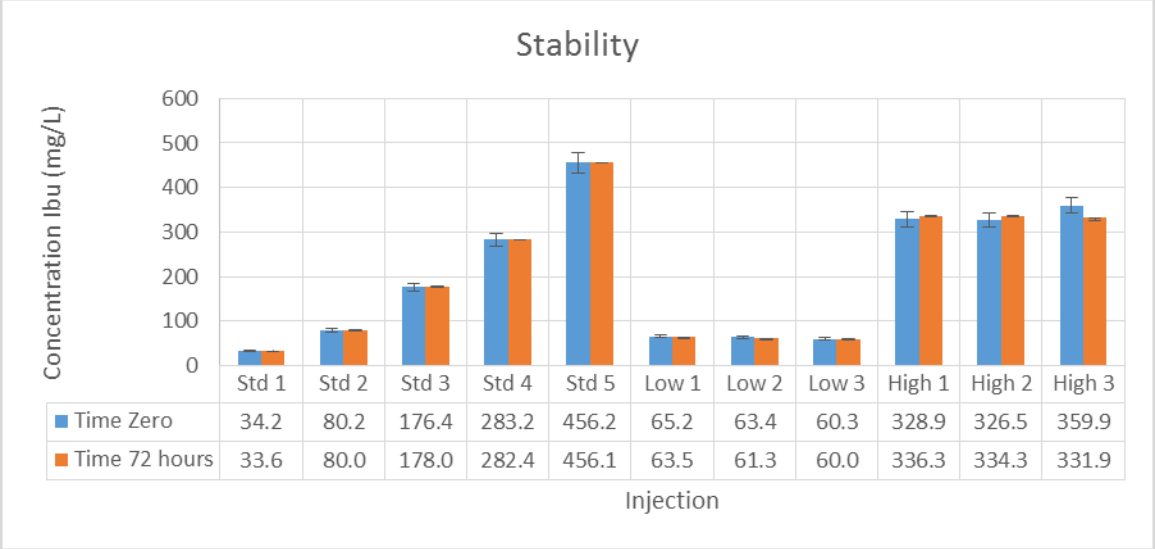


Figure 8: Chromatogram with all analytes originally included in the method.

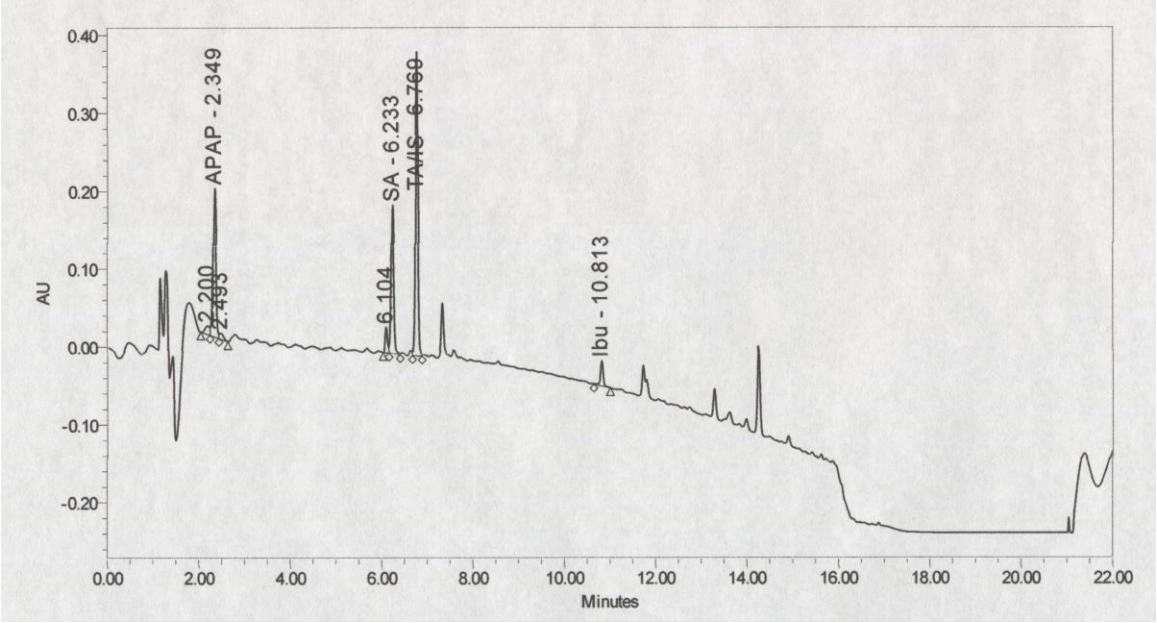


Figure 9: Chromatogram of the injection of naproxen, whose retention time is approximately 8.6 min.

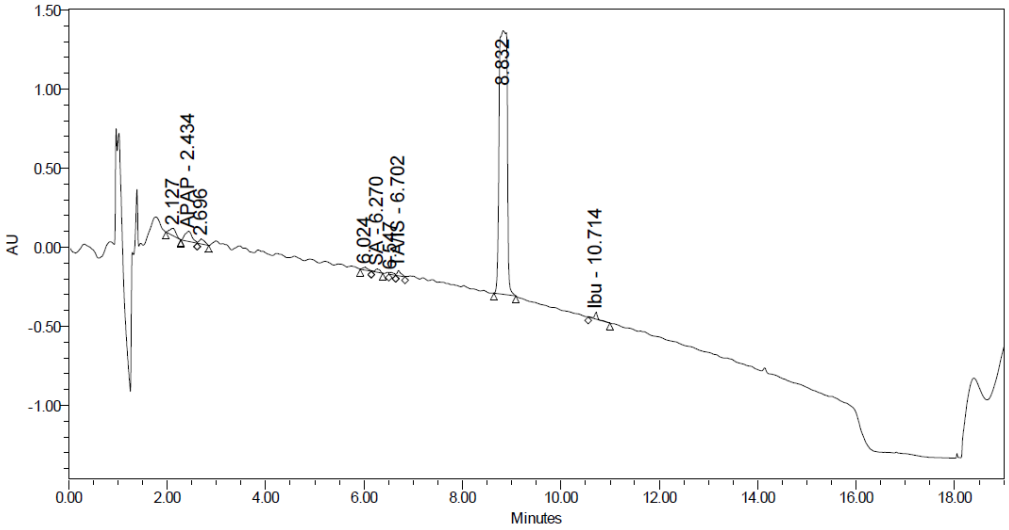


Figure 10: Chromatogram of the injection of other common drugs of abuse. Note the naproxen peak is still present at approximately 8.6 minutes.

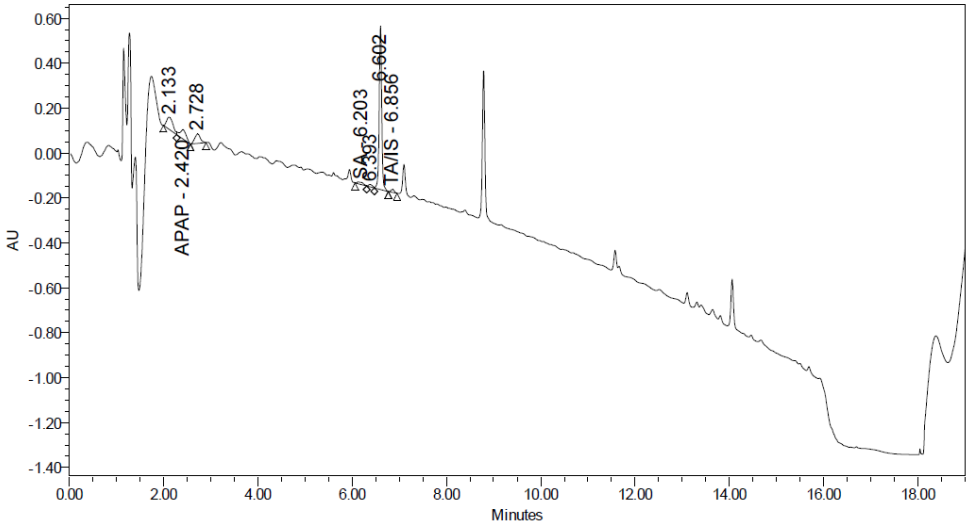


Figure 11: Chromatogram demonstrating the overlap of peaks from blood contamination and salicylic acid.

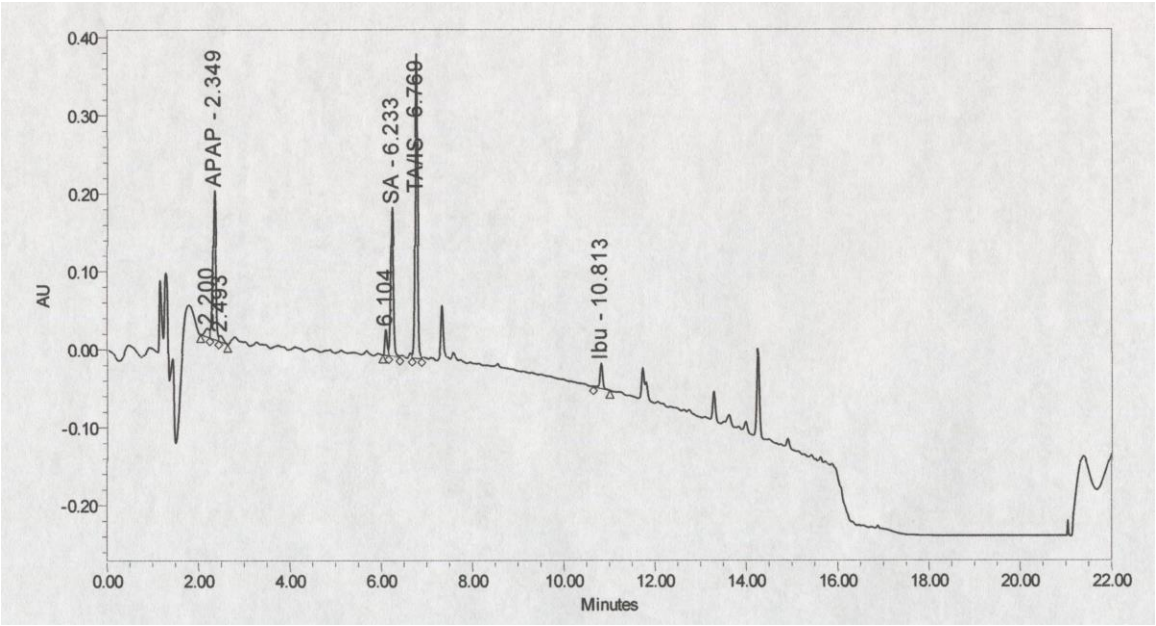


Figure 12: The interference peak resultant of an improperly stored column.

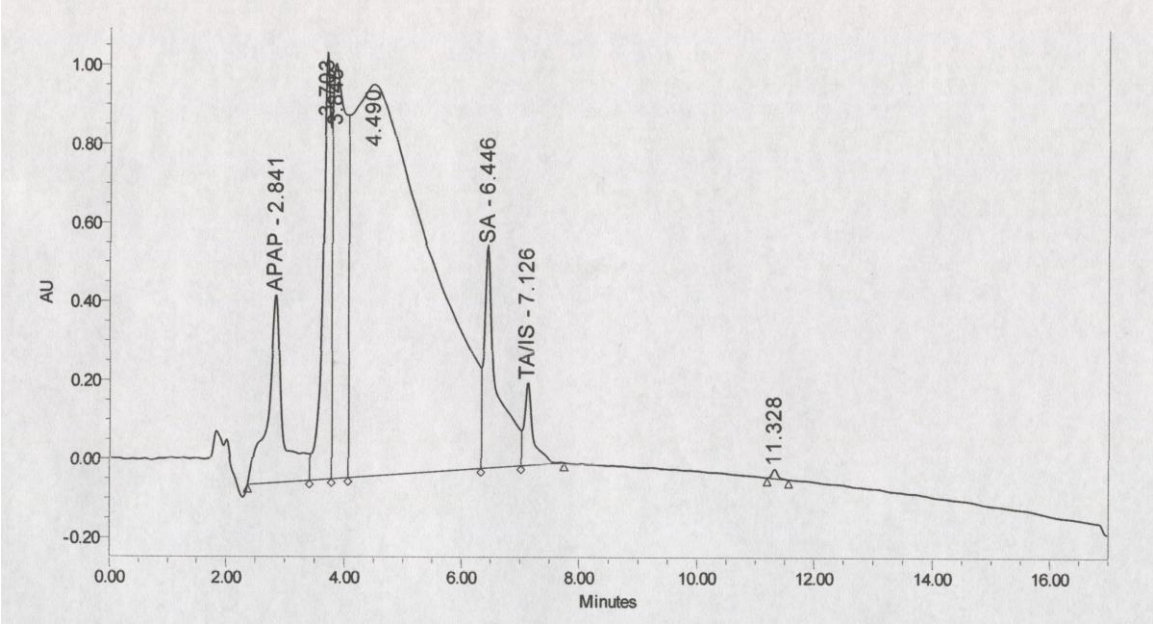
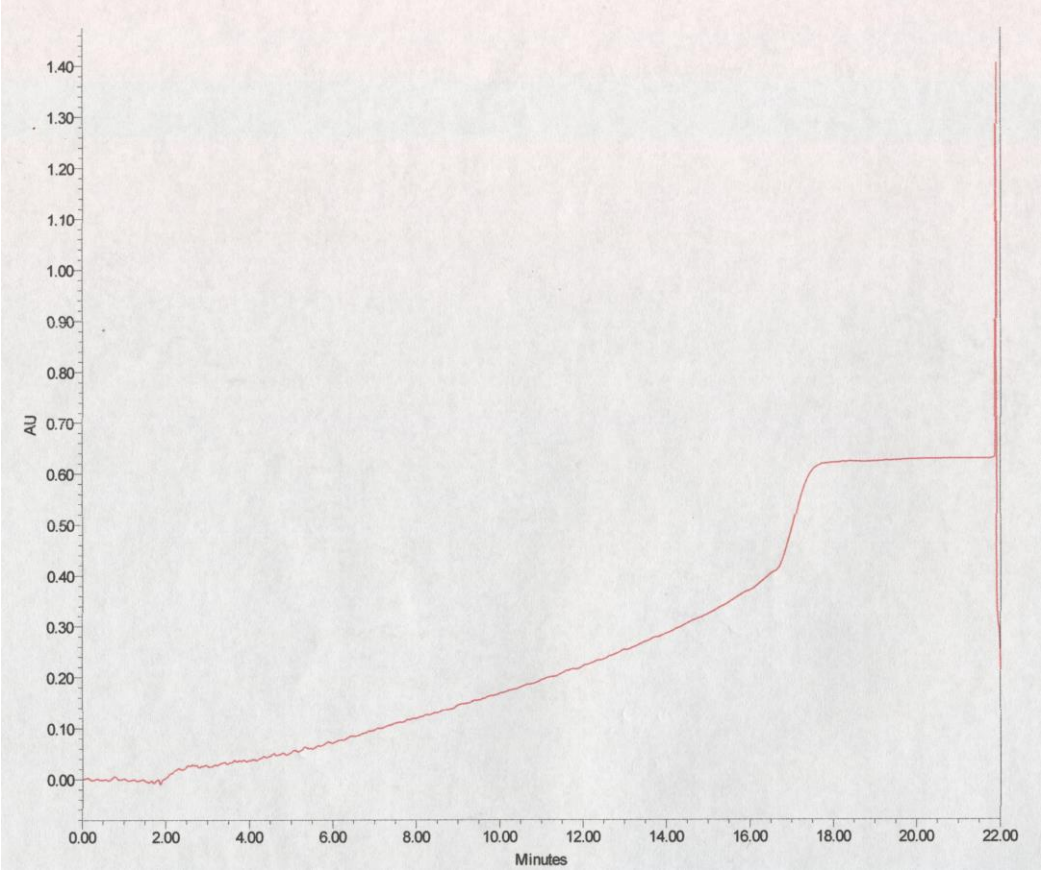


Figure 13: A run of just mobile phase with the addition of acetic acid to the organic mobile phase.



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