HPLC Determination of Amphetamine and Guanfacine

HonorsProject

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By

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Abstract:

Stimulant medications are most often prescribed for the treatment of attention deficit hyperactivity disorder (ADHD) and fetal alcohol syndrome (FAS). However, about 20% of the children taking stimulants do not have much symptom relief or they suffer from side effects. As a result, adjunctive therapy is often recommended. One approach is to prescribe Intuniv® (guanfacine) with an ADHD stimulant medication such as Adderall® (amphetamine). Many research studies report that Intuniv® given in combination with a stimulant leads to significant improvements in ADHD and impulsivity symptoms. Therefore, the purpose of this research is to develop an HPLC method to simultaneously determine the concentration of amphetamine and guanfacine in dosage form, which could then be used in the analysis of biological fluids.

Introduction:

The research being conducted in this project has not been the only kind done. There has been much research done on the basis of determination or identification of amphetamine and/or guanfacine in biological samples. Throughout all the research, there has been one common motive: develop a method of determination that is effective enough for guanfacine or amphetamine to be quantified in the biological samples. The various methods for determining guanfacine specifically include high-performance liquid chromatography[1,2], spectrofluorimetric determination[3], dried blood spot technology[4], liquid chromatography-tandem mass spectrometry[5], gas chromatography-mass spectrometry[6], and spectrophotometry[7]. The methods for amphetamine are gas chromatography-mass spectrometry[8-12], derivatization and gas chromatography-mass spectrometry[13], liquid chromatography-tandem mass spectrometry[14], liquid chromatography on a chiral stationary phase[15], solid phase extraction and ion-pair

chromatography-electrospray-tandem mass spectrometry[16], pipette tip solid-phase extraction and gas chromatography-mass spectrometry[17], and the use of a gold electrode[18]. In all of the previously mentioned methods, separation had been accomplished, though not all produced it simultaneously. However, obtaining the initial biological sample that is necessary for most of the methods is largely time consuming and difficult because access to the samples are extremely limited in part due to the unwillingness of institutions and medical professionals to share samples with one another[19].

Guanfacine, which is a non-stimulant, is thought to affect receptors in certain areas of the brain that strengthen working memory, reduce distraction, and improve attention and impulse control[20].

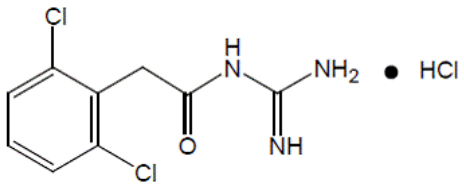


Figure 1. Structure of Guanfacine Hydrochloride

It is also the main component in Intuniv®. Intuniv is prescribed as a treatment to control symptoms of attention deficit hyperactivity disorder (ADHD). It can further be prescribed for high blood pressure.

Amphetamine is a synthetic stimulant of the central nervous system and is used to increase the synaptic activity of certain neurotransmitters[21]. Although it can be addictive, it is legally used as part of a treatment plan (along with guanfacine) in treating kids with ADHD. It works by restoring the balance of natural substances in the brain and helps with the ability to pay attention, focus, and remain still. Amphetamine is the drug used to

make Adderall® along with dextroamphetamine. It is not only prescribed for helping ADHD, but is also used to treat sleeping disorders and to help people stay awake[22,23].

Background/Purpose:

ADHD is the most commonly diagnosed neurobehavioral disorder in children. Symptoms can include hyperactivity, inability to control impulses, and inattention. This disorder mainly affects children, but can continue into adulthood[24]. It is clear the previously noted behaviors can impair and interfere with home life, social settings, and academic performance, but can be subdued through the use of medication. Over the past decade, there has been a dramatic increase in the diagnosis of ADHD. In 2005, according to the National Survey of Children's Health, the percentage of children ages 4-17 years who were diagnosed with ADHD increased from 7.8% to 9.5% during 2003-2007, indicating a 21.8% increase in four years. Furthermore, among children with current ADHD, 66.3% were taking medication. Thus, 4.8% of all children were taking medication (around 2.7 million)[25]. Although it can usually be discovered or diagnosed during a child's early life, how young is too young and are children being diagnosed with insufficient evaluation?

There are various types of ADHD: inattention, hyperactivity, and impulsivity[26]. The different types are categorized based on the severity of the symptoms observed. For example, a child with inattention may not like to do things the require sitting still, whereas, a child with hyperactivity, rarely remains seated if at all. Nonetheless, the symptoms are all similar, though may vary, as a person gets older. Symptoms can include but are not limited to anxiety, procrastination, mood swings, and depression. A specific cause of ADHD has not been identified, but researchers believe it can be attributed to several things such as heredity, poor nutrition, or chemical imbalances. It cannot be

prevented or cured, but spotting it early on can help ensure proper management of symptoms.

The management of symptoms can be done in the form of medication. Often, stimulants are prescribed to help control the hyperactive and impulsive behaviors, while also increasing the attention span. Some examples include Adderall®, Ritalin®, or Concerta®. Nonetheless, stimulants may not work for every person, or may not work favorably alone. Therefore, non-stimulant medication can be prescribed as well; this is known as adjunctive therapy. Some examples include Intuniv® and Strattera®. Medication is not the only form of treatment that can be used to help manage ADHD. Therapy such as special education or behavior modification can help children learn more efficiently in school and replace inappropriate behavior with good behavior.

For the research being conducted in this experiment, the drugs in focus will include Intuniv® and Adderall®. More specifically, HPLC or high performance liquid chromatography, will be used to simultaneously determine the concentrations of both drugs to further explore how well adjunctive therapy works and how they affect their purpose of treatment.

Grade Level	Adderall®
8th -graders	1.80%
10th- graders	4.40%
12th -graders	7.40%

Table 1 – Percentage of Students Prescribed Adderall® for ADHD

Adderall® is a commonly prescribed drug to help mange the affects of ADHD. It is a combination of amphetamine and dextroamphetamine. They are central nervous stimulants that affect chemicals in the brain and nerves that contribute to the

hyperactivity and impulsivity experienced by children who suffer from ADHD. It is available either as an immediate release tablet or extended release capsules. It works by increasing the activity of the neurotransmitters norepinephrine and dopamine in the brain[27]. It is generally well tolerated in children and can be effective in treating ADHD symptoms. Specifically, pharmaceutical amphetamines have the potential to improve brain development and nerve growth. Also, they improve stamina, endurance, and reaction time due to effluxion of dopamine in the central nervous system. Studies have also shown that long-term usage of amphetamines can decrease abnormalities in the structure of the brain as well as the function in several areas of the brain. Furthermore, approximately 80% of people who have used this stimulant have seen improvements in ADHD symptoms[28]. If children continue to use stimulant medication, they could have not only better relationships with their family, but also perform better in school and be less distracted.

On the other hand, Intuniv® is similar to Adderall® in its function to help treat ADHD, but it is not a stimulant drug. It is an extended-release form of guanfacine hydrochloride. It has the ability to strengthen parts of the brain that regulate attention and behavior, specifically the prefrontal cortical region[29]. In adjunctive therapy, gaunfacine is used in juxtaposition with stimulants such as amphetamines, to enhance therapeutic actions. While relatively new, it is currently approved and marketed in the U.S. as Intuniv®.

High performance liquid chromatography is basically an improved version of column chromatography. A solvent is forced through a column under high pressures, which makes it extremely quick and efficient. It also allows for the usage of much

smaller particles for the column packing material. This is important because the more small particles that are used increases the surface area which ultimately increases the probability for more interactions between the stationary phase and the mobile phase. This allows for better separation of the components in a mixture[30]. This technique is mostly used in the analytical chemistry field, and as given by the previous description, it is used to separate and identify each component in a mixture. This particular method is imperative to the study being conducted because it is used to "test the products and to detect the raw ingredient used to make them."[31] The biggest benefit from utilizing this method is obtaining a better understanding of the structure, impurities, and degradation, of a drug product that is not just limited to synthetic drugs, but also herbal medications.

Figure 2. Actual HPLC instrument used in the experiment

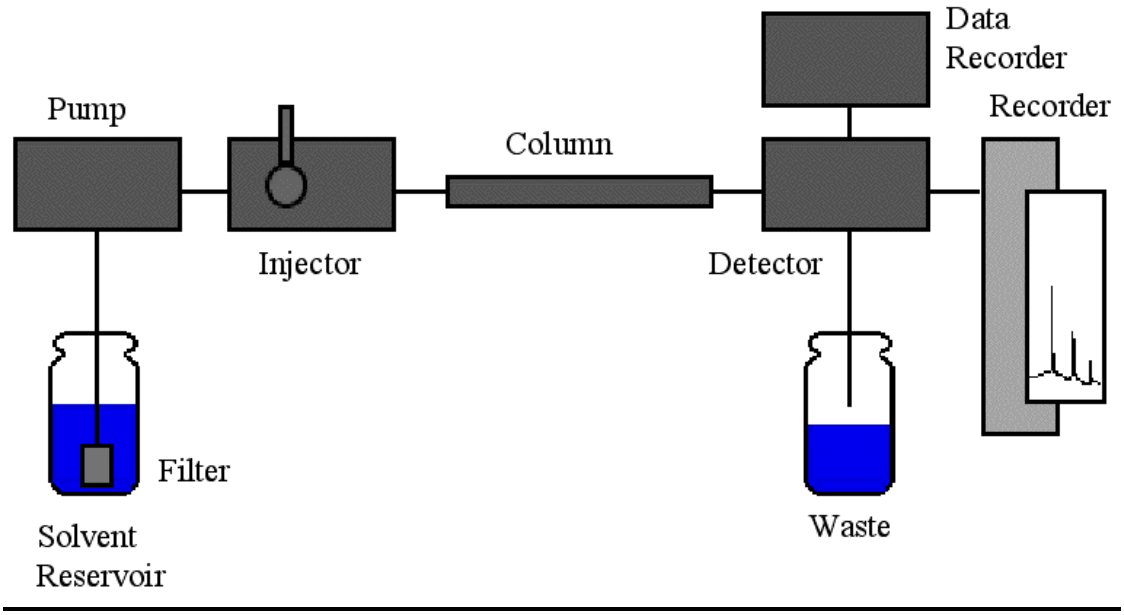


Figure 3. Schematic of the HPLC

Procedure:

Two different forms of guanfacine were used in the experiment: tablet and reference standard (powder).

Figure 4. Guanfacine tablet

First, the tablet was made into a solution in order for it to be analyzed by HPLC. The diluent was prepared by mixing 14 mL of water and 6 mL of acetonitrile in a beaker. Next, the guanfacine tablet (2 mg) was crushed into a fine powder. The powder was then dissolved in 10 mL of diluent. Once the solution was both prepared, it was stirred for about ten minutes using a magnetic stir plate due to its low solubility. Next, the dilutions were made: 1:5 (0.04 mg/mL), 1:10 (0.02 mg/mL), 1:20 (0.01 mg/mL), 1:50 (0.004

mg/mL), and 1:100 (0.002 mg/mL). Each dilution was sequentially injected three times into the HPLC instrument.

The guanfacine powder (reference standard) sample preparation began with preparing the solution it would be dissolved in, which is also the diluent. The solution was made by combining 42 mL of water and 18 mL of acetonitrile and mixing well. To dissolve the powder, 50 mL was used for the sample. What was left of the diluent was used to make five more dilutions: (1:5 (0.2 mg/mL), 1:10 (0.1 mg/mL), 1:20 (0.05 mg/mL), 1:50 (0.02 mg/mL), and 1:100 (0.01 mg/mL). As previously done, each sample was injected three times.

Experimental:

Chemicals and Reagents

The HPLC mobile phase utilized 80% acetonitrile and 20% 6.25 mM sodium phosphate with a pH adjusted to 3.2 using phosphoric acid (acetonitrile/sodium phosphate source: Fisher Scientific®). The source of both the reference standard and tablets is Sigma Aldrich®.

Equipment

The HPLC instrument was composed of a pump (Shimadzu DGU-20 AS), a column (Phenomenex Synergi 4u Hydro-RP 80A, 150 x 4.60 mm 4u micron), UV detector (Shimadzu SPD-20A), and Microsoft Excel data system.

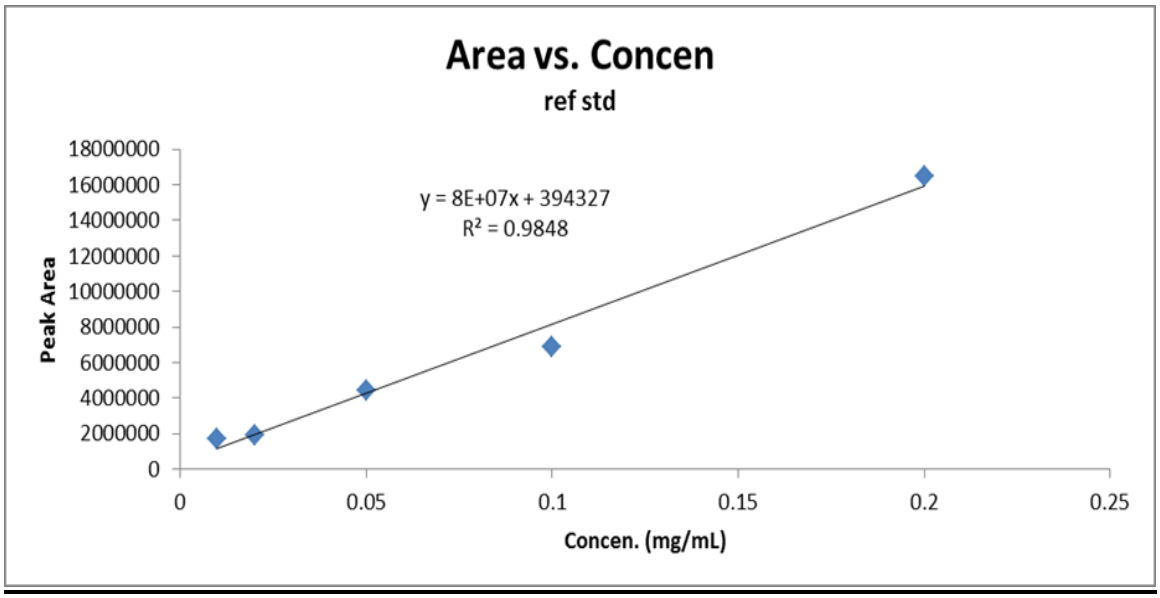
HPLC Method

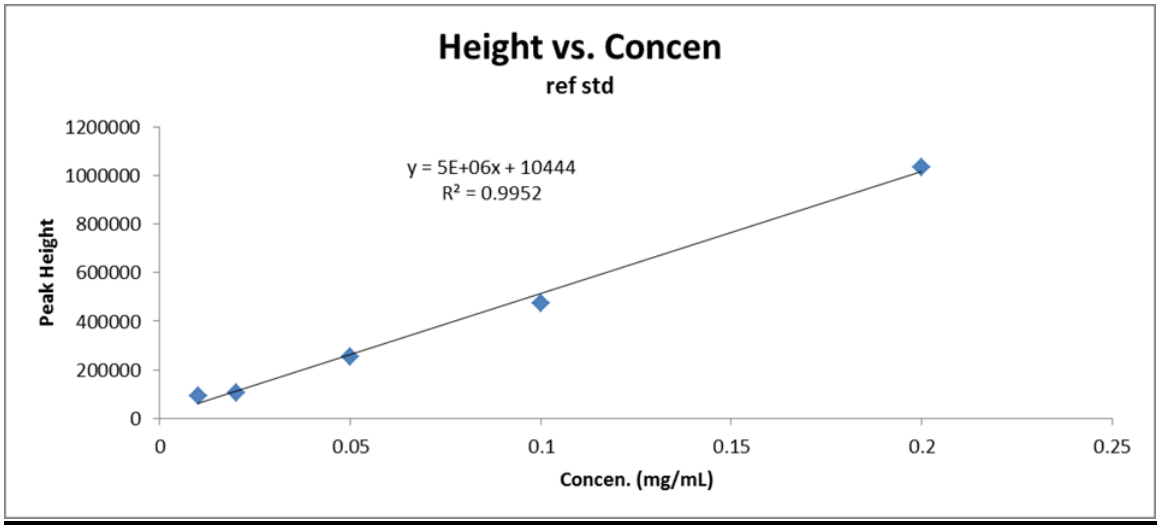
Through the use of a polar mobile phase of 80% acetonitrile and 6.25 mM sodium phosphate (pH 3.2), the solution was eluted through the column at a flow rate of 0.5 mL/min. The detector was set to a UV wavelength of 220 nm.

Data/Results:

		Tablet					
	Concen. (mg/mL)	Area	STDEV	RSD	Height	STDEV	RSD
	0.04	39648170			2125587		
		37795629			2043355		
		41002900			1955511		
Average		39482233	1610061.531	4.077939388	2041484.333	85053.4302	4.166254
	0.02	21663334			1073451		
		22073735			1223504		
		21918786			1112119		
Average		21885285	207241.364	0.946943867	1136358	77907.7823	6.855919
	0.01	4528187			447245		
		4812862			452864		
		4740558			454869		
Average		4693869	147969.1216	3.152391377	451659.3333	3952.184248	0.875037
	0.004	4279387			190362		
		4439124			210064		
		3908031			212702		
Average		4208847.333	272482.7097	6.474045935	204376	12207.94446	5.973277
	0.002	1777240			95516		
		1869122			84996		
		1883708			87636		
Average		1843356.667	57721.29639	3.131314598	89382.66667	5473.183108	6.123316

		Reference Std					
	Concen. (mg/mL)	Area	STDEV	RSD	Height	STDEV	RSD
	0.2	16181918			1037437		
		16507459			962014		
		16758081			1104165		
Average		16482486	288892.1748	1.752722101	1034538.667	71119.80703	6.874543149
	0.1	5799404			423935		
		7252052			450998		
		7643544			551735		
Average		6898333.333	971622.7438	14.08489119	475556	67346.35493	14.16160346
	0.05	4319936			251576		
		4485695			261486		
		4575023			245762		
Average		4460218	129437.8327	2.90205171	252941.3333	7950.417935	3.143186536
	0.02	1754450			101863		
		2018195			110454		
		1969170			100124		
Average		1913938.333	140279.218	7.329348892	104147	5530.79714	5.310567889
	0.01	2677565			149278		
		1277453			66960		
		1205821			63778		
Average		1720279.667	829806.7202	48.23673361	93338.66667	48471.00207	51.93024906





		Concen. (mg/mL)	Area	STDEV	RSD	Height	STDEV	RSD
		0.013	1153287			54290		
			1150587			56611		
			1188452			61300		
	Average		1164108.667	21125.12505	1.814703872	57400.33333	3571.037711	6.221283926
		0.033	2630823			139739		
			2729841			148323		
			2782506			146640		
	Average		2714390	77012.87667	2.8372075	144900.6667	4548.650826	3.139151068
		0.066	6067193			292853		
			6402199			340579		
			6508095			322012		
	Average		6325829	230158.4803	3.638392381	318481.3333	24058.09582	7.554004991

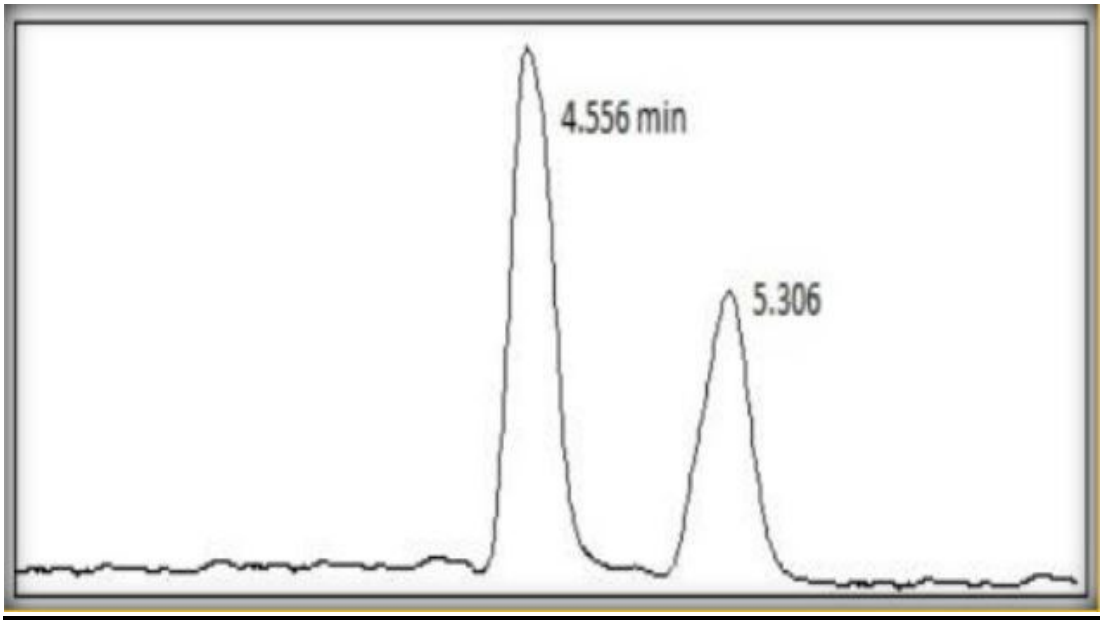


Figure 5 – Guanfacine (0.5 mg/mL) elutes at 4.556 minutes and amphetamine (0.6 mg/mL) elutes at 5.306 min.

Discussion:

The first set of experimental data was collected from the tablet form of guanfacine. For each of the concentrations, the standard deviation (STDEV) and the relative standard deviation (RSD) was calculated. The calculated values were then used to analyze the data along with the error in the data. The values were suitable for some of the concentrations while others were not, possibly due to some outliers. Because of this error, some modifications need to take place so all the numbers will be more accurate.

The second set of experimental data referred to the reference standard used in the experiment. One concern regarding the table that stood out was the high value of the RSD at 48.2 with the concentration of 0.01. In order to check for possible outliers, a Q test was performed. Once the test was complete, the value calculated was compared to a literature value at the 99% confidence level. While the RSD was abnormally high, it fell within a specified range and was thus determined not to be an outlier. Furthermore, the actual calculation of both the standard deviation and the relative standard deviation helped

examine the accuracy and precision of the measurements taken in the table; it is taught that good precision does not always mean good accuracy. The standard deviation is an indicator of precision based on the reliability of the experimental results while RSD indicates how widely spread the values are from the average. If the standard deviation is high, the results will be seemingly unreliable. It is determined by first calculating the mean. Then, for each number, subtract the mean and square the result. Finally, work out the average of those squared differences. In general terms, precision applies to how well the same measurement was delivered repeatedly, while accuracy applies to how close the results are to the actual measurement. As far as precision is concerned, the RSD for the same concentration repeated in multiples should be below 2%. With a decreased number of samples, a precision of 5-10% RSD is generally accepted. Referring to accuracy, the percent recovery should be within 90-110% (for spiked samples; this is called spiked recovery). These would represent the concentrations not present on the calibration curve. When looking at the linearity of the graphs, it is important to note the correlation coefficient, which a measurement of how accurate a linear relationship is. Ideally, 0.98 or better is best. For both curves comparing the height and areas, the correlation coefficients were within an acceptable range, indicating good measurements. In continuation of the analysis, the height and area were observed as being dependent on the concentration. The calibration curve showed how the height was more accurate than the area. Also, the limit of quantification (LOQ), which is defined as the lowest concentration at which the analyte can be detected (concentrations lower are not within the linear range), was determined to be the 1:50 (0.02 mg/mL) dilution for the reference standard.

The third set of data also concerned the reference standard. The particular set of data was of concentrations between the previously recorded data to verify if the height or area was better use for the calculation of the concentration. This data was also used to calculate the expected concentrations using the calibrations curves. The calculated concentrations were lower than normal, which indicated that some alterations could be done to produce better results.

Finally, Figure 5 is an illustration of what an ideal graph of the concentration of guanfacine and amphetamine would look like if we were successful in simultaneously determining their concentrations.

Future Work:

In the future, it would be nice to develop an HPLC method to simultaneously determine guanfacine and amphetamine. The first priority is to find a method to be used for amphetamine that is close in relation to the method used for guanfacine. However, the method for guanfacine needs to be validated and enhanced for acceptable accuracy and precision.

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