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STANDARDIZING AND IMPROVING A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR CYCLOSPORIN

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Bethany D. Annett

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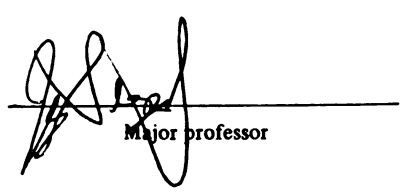

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STANDARDIZING AND IMPROVING A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR CYCLOSPORIN.

By

Bethany D. Annett

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ABSTRACT

STANDARDIZING AND IMPROVING A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD FOR CYCLOSPORIN

By

Bethany D. Annett

Forensic toxicologists are often faced with the need to standardize analytical methods for a variety of drugs. New drugs are being created, or newly abused all the time, and toxicologists must stay current in their abilities to test for these drugs. Even current methods may need to be standardized or improved to be in compliance with increasing regulations. In this project, a method to test for cyclosporin, using high performance liquid chromatography (HPLC), will be improved and standardized, serving as a model for the standardization and refinement process.

Many different instrument conditions were experimented with, including the HPLC column, oven temperature, flow rate, and mobile phase. Once the instrument conditions were established, extraction methods were performed and compared to determine which was the most effective. Improvement of the method occurred after optimization of both the extraction procedure and instrument conditions. Once the method is optimized, standardization can occur.

The standardization of the method includes determining the linearity of the method, the limit of detection – limit of quantification, and precision, both run-to-run, and with-in run. By following the pattern set forth in this project, the testing for any drug can be optimized and standardized.

I would like to dedicate this to my parents. Without their support and encouragement, this would never have been possible.

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1.0 Introduction

Forensic toxicologists are faced with ever-increasing demands for more sensitive and specific methods to detect an increasing number of drugs. For example, the "date rape drugs" rohypnol and GHB have recently come into use. Forensic toxicologists have had to develop and standardize methods to test for these drugs. Modern instrumental analytical chemistry and the rapid advances in analytical techniques offer a challenge as well as an opportunity to utilize these resources for forensic analysis. By adapting these techniques to the analysis of biological specimens, toxicologists will be able to provide more accurate and precise data to those who are dealing with:

1. the cause of poisonings;
2. improving therapeutic approaches using currently available drugs;
3. the development of new drugs and their pharmacology;
4. understanding the implications of substances detected in biological specimens during medico-legal investigations.

Hence, selection, development, and evaluation of methods and instrumentation are critical steps in establishing and ensuring the quality of forensic laboratory services. Continual improvement of instrumentation and methodology should be an integral on-going function of all laboratories.

As an example in an associated field, the drug cyclosporin is an immunosuppressive agent that is currently being given to individuals who have received kidney, liver, heart, or other organ transplants. Cyclosporin acts specifically on the cells involved in graft rejection, rather than as a general

suppressant of the entire immune system. Once the individual has received the transplant, and the cyclosporin is administered, it is important to monitor the concentration of cyclosporin in a person's blood because there are many dose-dependant side effects. While an extremely high level can be toxic, too low a concentration can result in rejection of the transplant. As the number of transplants has increased, the use of cyclosporin has also increased, along with the need for improved analytical detection. The laboratory was currently sending cyclosporin tests to Mayo laboratories for analysis. This project was designed to enable the laboratory to perform these tests in-house, rather than paying someone else to do them.

The purpose of this project is to identify specific improvement and standardization criteria for a high-pressure liquid chromatographic (HPLC) procedure for cyclosporin, which will serve as a general model that identifies a process for the development, improvement and standardization of forensic toxicology procedures.

1.1 Project Description

The project consists of two main parts. The first part involves the instrumentation and the extraction method. The instrument, which provides for the detection and quantification of cyclosporin, must first be selected based on sensitivity, availability and practicality. The instrument conditions must then be optimized. When using an HPLC, as in this case, a column must be chosen, and then an appropriate mobile phase, flow rate, and oven temperature. The goal is to find a combination of column, mobile phase, and other instrument conditions

that provide a short run time with adequate peak separation. Once the instrument is optimized, an extraction method must be chosen. The method will be chosen based on the ease of the extraction, the amount of time involved, and the recovery of the drug.

The second part consists of the improvement and standardization of the method selected. Improvement of the method can involve changes in either the instrument conditions or the extraction method. Once the final method is optimized, it must be standardized. This includes the linearity, limit of detection/limit of quantification, carryover, correlation with another method, etc.

The purpose of this project is to serve as a model for other forensic toxicology laboratories to follow in the development, standardization and improvement of analytical methods. The necessary use of new methods may be prompted because of new drugs being created and popularized, or because of higher standards being placed upon the laboratories. In some cases, methods are already in place, but have not been properly standardized due to a lack of regulation. In such situations, the second half of this project is applicable to bring the methods up to standard.

2.0 Literature Review

2.1 Extraction

Several articles, instruction manuals and standard operating procedures deal with the extraction methods. Currently, both solid phase and liquid-liquid phase extraction methods are being utilized for cyclosporin analysis. The liquid-liquid extractions use methyl t-butyl ether (MTBE) or diethyl ether to extract the cyclosporin from blood that has been acidified (1,5,7). The Clean-Up® solid phase extraction uses a two-column system (3,6). The Waters Oasis® columns contain a copolymer with a hydrophilic-lypophilic balance (8). The Bio-Rad® solid phase extraction uses a silica clean up column (2). Three different types of solid phase extractions and two liquid-liquid phase extractions were tested and compared for recovery, ease of extraction, and length of extraction.

2.2 Instrumentation

Several articles deal with the choice of instrumentation when testing for cyclosporin. The quickest and easiest methods utilize immunoassay techniques (9,10,11). Using the HPLC is a longer process, and involves a more sophisticated extraction procedure, but is more specific, measuring only the parent drug (9,10,11). Because HPLC is the most widely used instrumentation, due to its sensitivity and lack of interferences, it will be the instrumentation utilized in this research.

2.3 Standardization

Standardization of the method was performed following the guidelines set forth in the "Tietz Textbook of Clinical Chemistry Chapter 15, Selection and

Evaluation of Methods" (4). This gives different criteria for both choosing and standardizing laboratory methods. Following these guidelines assures that the method is standardized in a way that is approved by the National Committee for Clinical Laboratory Standards (NCCLS).

Several of the methods previously mentioned might have worked without major modifications. However, since the standardization was necessary regardless of which method was chosen, and in keeping with the purpose of this project, an attempt was made to not simply copy another method, but to also improve that method.

3.0 Background Information

There are two main aspects to the first part of this project – the extraction and the instrumentation. Background information is provided in this section regarding both extractions and HPLC instrumentation.

3.1 Extraction

There are two different types of extractions that can be performed – solid phase and liquid phase. At least one of each of these types was utilized during the course of this project. There are some inherent advantages and disadvantages to each type of extraction, which will be discussed in further detail.

3.1a Solid Phase Extraction

During a solid phase extraction, a column containing a sorbent bed is used to isolate the drug from the solvent it is in. The drug is extracted from whole blood into an organic solvent. Because of its physical properties, the drug is more attracted to the solvent than the blood and more attracted to the column than the solvent. Thus, when the solvent passes through the column, the drug adheres to the column, while other components pass through without being retained. The column is then rinsed with water or selective buffers which clear away other contaminants while the drug is retained. The column is then dried to remove all traces of the volatile solvents. A final eluting solvent is used to remove the drug from the column and dissolve it in the solvent.

The first solid phase extraction used was from Bio-Rad®. This column is a bonded phase clean up column containing a reversed phase silica packing. This extraction calls for first adding the whole blood sample to water and internal

standard in 30% acetonitrile. This lyses the red blood cells and the drug is released into the supernatant. The solution is then vortexed and centrifuged and the supernatant is applied to the prepared column. The columns were prepared by adding first 95% ethyl alcohol, and then 15% acetonitrile. Once the supernatant has passed through the column, it is rinsed with15% acetonitrile to remove proteins, and 50% acetonitrile to remove less hydrophobic compounds. Cyclosporin is eluted with 95% alcohol, and the eluate is then diluted with an acidic dilution reagent to reduce the percentage of alcohol. This eluate is then washed with hexane to remove lipids and other more hydrophobic compounds. The cleaned eluate is then injected on the HPLC (2).

The second solid phase extraction tested was the Clean-Up® extraction method. This uses a C18 column for extraction, and then a silica column to filter. The C18 column is widely used for extracting non-polar or neutral compounds because of its non-selective nature. The sample is mixed with internal standard and 9:1 acetonitrile:methanol, vortexed, and centrifuged. This extracts the cyclosporin out of the blood, and into the solvent. The column is prepared by washing with acetonitrile and 2:8 acetonitrile:water. Without letting the column dry out, the sample is added. The column is then washed with 7:3 methanol: water to remove more hydrophilic compounds and 1:99 acetone:hexane to remove less hydrophilic compounds. The cyclosporin is eluted with 75:25 ethyl acetate:isopropanol. The eluate is filtered through a silica column to remove hydrophilic compounds and then evaporated. The sample is reconstituted with mobile phase, and is ready to be injected on the HPLC (3).

The last solid phase extraction tested was the Waters Oasis® columns. These contain a "unique patent-pending sorbent, a copolymer designed to have Hydrophilic-Lipophilic balance that gives high and reproducible recoveries for acidic, basic, and neutral compounds" (8). The sample and internal standard are mixed with a precipitating reagent, vortexed, and centrifuged. The columns are activated with 95% ethanol, then water, then repeated. The spun sample is added to the column. Once they have drained, the columns are washed with water, then 50:50 acetonitrile:water. These steps remove the more hydrophilic compounds. Cyclosporin is extracted from the column using ethanol, which is then dried down. Mobile phase is used to reconstitute the sample, which is then rinsed with heptane to remove lipids. The sample is then ready to be injected on the HPLC (8).

In general, solid phase extractions take less time than liquid phase extractions. They also tend to remove more impurities than liquid phase extractions. In some cases, they also yield a better recovery. Depending on the method, however, they require more attention than liquid phase extractions.

3.1b Liquid Phase Extraction

A liquid-liquid extraction consists of removing the drug from the blood and into an organic solvent by changing the pH. The acidification of the blood causes the drug to move to the solvent. The first extraction uses MTBE (methyl t-butyl ether) as the organic solvent. This extraction begins with acidifying the blood with HCl, mixing, and adding MTBE. The mixture is vortexed, mixed, and the two phases are separated by centrifugation. Cyclosporin is extracted from the blood into the

MTBE. The solvent is then washed with NaOH by again mixing and centrifuging. This helps to remove other impurities. The MTBE is then dried down and the sample is reconstituted with mobile phase and rinsed with heptane to remove lipids (5).

The second extraction method used diethyl ether, and all of the volumes used in the method were cut in half due to available glassware and centrifuge capabilities. The whole blood sample has internal standard added to it, and HCl. The sample is mixed well, and diethyl ether is added. The samples are mixed, and then centrifuged to separate the two phases. The organic phase is transferred to new tubes and dried down. The residue is redissolved in HCl, and mixed with hexane. The hexane is removed, and the aqueous phase has NaOH added, along with diethyl ether. The sample is mixed and centrifuged. The organic phase is transferred to a new tube and evaporated. The sample is reconstituted with mobile phase and is ready to inject on the HPLC (7).

This same extraction was performed using MTBE in place of diethyl ether. MTBE is less volatile, so the extraction takes longer, but is much less dangerous to use and store.

In general, liquid phase extractions take longer than solid phase ones, however, they are often less time intensive, do not take as much attention, and do not require as many tech hours. Liquid phase extractions in general do not remove as many impurities as solid phase extractions.

3.2 Instrumentation

The HPLC was the instrument chosen for this project, however, there are several other instruments that could have been utilized. Several of these instruments were available in the laboratory, but the HPLC was considered more practical for various reasons identified in this section. Background information on how an HPLC works is also provided.

3.2a Overview of available instrumentation

There are many different instruments capable of testing for the drug cyclosporin. They include immunoassay instruments, HPLC, and GCMS. All three types of instrumentation were available in this laboratory. The immunoassay instruments include AxSYM®, TDx® (which is fluorescence-polarization immunoassay, or FPIA), Emit® (enzyme-multiplied immunoassay), and RIA (radioimmunoassay). These work by using the principle of competitive binding between the drug and drug that has been labeled with either tracers, radioactive, or enzymes. The advantages to using immunoassay test include a much shorter extraction, if any at all, a shorter run time, and the ability to test many samples at once. The disadvantages include a much higher cross-reactivity with cyclosporin metabolites, which causes the values to be inflated.

As a result of this lack of sensitivity, and the potential for interferences, these instruments were not utilized in this project. The laboratory requires instrumentation that provides an accurate quantitative value of cyclosporin without significant interferences.

In contrast, both HPLC and GCMS provide adequate sensitivity, minimal interferences, and allow for the incorporation of an internal standard. This increases the precision and accuracy of the test by lessening the importance of sample size or injection volume. The GCMS, however, was not chosen in part because of its high sensitivity. Extraction processes usually take longer for the GCMS because the samples need to be cleaner with fewer impurities. In general, it is overkill to use a GCMS for this test. It provides information that is not needed, and is more expensive to run. It was not found in the literature where any one used GCMS to analyze for cyclosporin. The HPLC was the ideal instrument for this test because it entails a relatively quick extraction process, a high degree of sensitivity, a short enough run time, and provides a quantitative value for cyclosporin.

During the course of this project two different HPLC instruments were used. At the start of the project, a Waters 501 HPLC was used. This was connected to a chart recorder. During this time, a new HPLC, a Shimadzu with auto-sampler was purchased and set up. Once the new instrument was ready, research transferred over to it for several reasons. First, having the auto-sampler means that the technician no longer needs to return to the instrument every five or ten minutes to make the next injection. The computer is programmed with the sequence, and the samples are automatically injected. Second, the computer calibrates using the calibrator and internal standard and calculates the value of each sample automatically, and prints out a report. All of these values can be saved on the computer, and printed out again later if necessary. Third, the dual

set of pumps makes changing the ratio of the mobile phase much easier. This is especially important while trying to optimize a method. Rather than making up many mobile phases of differing ratios, one pump is hooked up to one component of the mobile phase, and the other pump, to the other component. Then the pumps can be programmed to pump at different speeds to achieve the desired ratio.

3.2b Theory and Practice of HPLC

The HPLC consists of an injector, pumps, column, mobile phase, oven, and detector.

The injector introduces the sample into the HPLC system. This is either done by hand with a syringe, or automated with an auto-sampler. The volume introduced is important and should be reproducible, although this is not as critical when an internal standard is used, as in this project. Using the simplest method, a syringe injects the sample through an elastomeric septum. In stop-flow injection, the flow is stopped and the sample injected directly into the column using a syringe. The most widely used method makes use of sampling loops. The sampling loops are made to contain various volumes of sample, from 5 to 500μL. The sample is loaded into the loop, and then it is injected to the column. This allows the sample to be introduced to the column without depressurizing the system.

There are several different types of pumps available for use with HPLC. They include reciprocating pumps, which are the most common, syringe type pumps, and constant pressure pumps. The reciprocating pumps use a motor-driven

piston to pump mobile phase into the column. On the backstroke, mobile phase is pulled in, and on the forward stroke, it is driven out to the column. These have the advantage of being able to achieve a wide range of flow rates. Dual and triple head pumps consist of identical units, which are 120 or 180 degrees out of phase. This type of pump system is significantly smoother.

Syringe type pumps, or displacement pumps, have a very small capacity, and are therefore most suited to small bore columns. They consist of a large syringe type reservoir, with a plunger that is activated by a motorized lead screw. The flow rate can be controlled by changing the voltage on the motor.

Constant pressure pumps use pressure from a gas cylinder to drive the mobile phase through the column. In order to generate high liquid pressures, a low-pressure gas source is needed. The solvent chamber has a low capacity, but a valve arrangement allows for rapid refill, and provides continuous mobile phase flow rates.

HPLC columns are usually made of stainless steel tubing, are 10-30cm long, with an inside diameter of 4-10mm and packed with 5 or 10μm particles. The newer micro-columns are 3-7cm long, with an inside diameter of 1-5mm and column packings of 3 or 5μm. The most common packing material is silica particles with highly uniform diameters. It is often coated with a thin organic film, or stationary phase. Other packing materials include ion-exchange resins, alumina particles, and porous-polymer particles. The stationary phase can be bonded to the packing by covalent bonds, which is called bonded-phase packing. A range of polarities is available, depending on what organic functional group is

bonded to the silica surface. The other type of packing is liquid-liquid, where the stationary phase is held on by physical adsorption. Historically, liquid-liquid was almost exclusively used, but now bonded-phase methods are by far the most popular. Bonded-phase packings are significantly more stable, and thus can be used longer.

There are two types of columns that are distinguished by the relative polarities of the mobile and stationary phases. Early work focused on using a polar stationary phase with a non-polar mobile phase. This is now referred to as a normal-phase column. Reverse phase columns have a non-polar stationary phase, and use a polar mobile phase. Using a normal phase column, the least polar components are eluted first. Increasing the polarity of the mobile phase decreases the elution time. With reverse phase, the most polar component elutes first, and increasing the polarity of the mobile phase increases the elution time. If a polar analyte is being tested for, then a reverse phase column will result in a shorter run time.

Choosing a column and mobile phase are two of the most important steps taken when setting up a new procedure. As a rule, separation is achieved by using a column on which the stationary phase polarity is similar to that of the analyte and a mobile phase which has a very different polarity. If the polarity of the analyte and the stationary phase are too similar, however, the retention times become very long. If the polarity of the analyte and the mobile phases are similar and the stationary phase is different, then the retention times are too short for practical use.

Guard columns are often used in front of the column. This short column helps protect the analytical column and increase its lifespan by removing larger particles and impurities before they can enter the column. The composition is similar to that of the analytical column, but the particle size should be larger to minimize the pressure change.

In the majority of cases, temperature is not a critical variable, and columns are kept at room temperature. Higher temperatures, however, produce faster run times and sharper peaks, and in order to optimize some runs, an oven is needed to heat the column up to a specific temperature. For example, cyclosporin reacts with the packing material in all columns, thereby causing some retention, which results in a very broad peak. Heating up the column eliminates this problem and results in a shorter run time.

There are many different detectors that can be attached to an HPLC. They include Ultra-Violet, Refractive Index, Fluorescent, Electrochemical, Mass Spectroscopy, and Light Scattering.

Ultra-Violet detectors measure the ability of a sample to absorb light. This can be done in several different ways. The sample flows between the UV source and the detector, and the amount of light absorbed is measured. The simplest detectors use a mercury lamp as the source and measure at the 254nm wavelength. Deuterium or tungsten filament sources can also be used. A fixed wavelength detector measures at one wavelength. Variable wavelength can detect over a range of wavelengths, but only measures one at a time. Diode

Array detectors measure several wavelengths at once. Ultra-Violet detectors are some of the most commonly used.

Refractive index detectors measure the ability of a sample to bend or refract light. One channel detects only the mobile phase, while the other detects the mobile phase passing through the column. The sample eluting from the column bends the light, and the difference between the two sides is measured.

Fluorescent detectors measure how a compound absorbs and then re-emits light at given wavelengths. The excitation source passes through the flow-cell to a photodetector while a monochromator measures the emission wavelengths.

Using mass spectroscopy detectors, the sample is first ionized, either by electron impact (EI) where an electron current is used, or chemical ionization (CI) where ionized gas is used to remove electrons from the eluate. The ionized sample is then passed through a mass analyzer, and the ion current is detected. The largest problem in connecting these two instruments is the large amount of solvent volume required for the HPLC and the vacuum requirements for the MS. Several different connecting units have been developed in order to solve this problem. There are several different commercially available interfaces. Some of these split the eluent, or evaporate the solvent, so that only a small portion is introduced to the MS.

Light-scattering detectors have the column eluate pass through a laser beam. When a laser strikes particles in solution, some light is scattered, reflected, absorbed, or transmitted. Light–scattering detectors measure the light that is transmitted or scattered, depending on the particular detector.

4.0 Methods and Materials

In order to identify the optimal instrument conditions and extraction, it was necessary to go through several sequences of optimizing both the instrument and the extraction. Once the final method is established, the improvement and standardization stages can occur.

4.1 Instrumentation and Extraction

Two different instruments were utilized in this project. The first was a Waters 501 HPLC with a variable wavelength detector. The second was a Shimadzu HPLC with auto-sampler and diode array detector. When the project was initiated, only the Waters instrument was available. Once the Shimadzu HPLC became available, the research transferred over to that instrument due to its increased capabilities and conveniences.

4.1a Waters HPLC with Bio-Rad® mini column and Bio-Rad® extraction

Using the Waters HPLC, the first HPLC column tested was the Bio-Rad® 30x4.6mm RP mini-column. This is a reverse phase column with silica packing. The recommended mobile phase of 50% acetonitrile in ammonium phosphate buffer was utilized first. The pH of the buffer is between 6.0 and 6.1. The flow rate is 1.2mL/min and the column temperature is at 70°C. A test solution consisting of 800ng/mL of both cyclosporin A and C was prepared, and 100µl was injected into the HPLC.

The cyclosporin A and C peaks co-eluted and could not be separated. In order to increase the peak separation, more ammonium hydroxide was added to the buffer to increase the pH of the mobile phase. Buffers with a pH of 7.3 and

8.3 were both tried, and although the separation was improved, especially with the pH 8.3, baseline was still not achieved between the two peaks.

A new mobile phase, 1:1:1 methanol:water:acetonitrile, was utilized in an attempt to improve the separation. Cyclosporin A and C did not separate, with the peaks overlapping one another.

As a result of these runs, it was determined to utilize the 50% acetonitrile in ammonium phosphate buffer with a pH of 8.3, and to begin focusing on extraction methods. Although the chromatography was not ideal, it was adequate, and would be improved later.

The Bio-Rad® solid phase extraction method, using a bonded phase cleanup column, was tested first. Several spiked controls were extracted and then injected on the HPLC, using the mobile phase of 50% acetonitrile in ammonium phosphate buffer, with the pH of the buffer at 8.3. No drug was detected. The extraction was repeated several times, and each time no drug was detected. Communication with Bio-Rad® personnel identified that this lot of extraction columns were not dependable several months before their expiration date. In addition, it was discovered that the company had terminated manufacture of these columns. As a result, the search for an effective extraction continued, and use of the Bio-Rad® RP mini HPLC column was reviewed as well.

4.1b Waters HPLC with Prodigy column and Clean-Up® extraction

Before performing additional experiments with extractions, and in an attempt to reduce run time and increase peak separation, the Prodigy 5u C8 (150 x 4.6mm) HPLC column from Phenomenex was used with a mobile phase of 1:1:1

acetonitrile:methanol:water. This mobile phase was recommended in the Clean-Up® extraction method. With a flow rate of 1.0mL/min, and the oven at 70°C, the cyclosporin A and C peaks could not be separated. A mobile phase of 1:1:3 acetonitrile:methanol:water was tried with a flow rate of 0.6mL/min. Again, the peaks could not be separated. A mobile phase of 7:3 acetonitrile:water was utilized next with a flow rate of 1.0mL/min with the oven at 70°C. The cyclosporin A and C peaks were nicely separated, although not quite all the way to the baseline.

Having further optimized the instrument, the research again focused on extraction. Several standards were extracted using the Clean-Up® solid phase extraction columns and procedure. Standards were made by spiking whole blood with a stock solution of cyclosporin A to achieve concentrations of 500 and 2000ng/mL. These standards were extracted after adding 200µl of internal standard (cyclosporin C), for a concentration of 200ng/mL.

Using the previously chosen mobile phase (7:3 acetonitrile:water) and instrument conditions (flow 1.0mL/min, and oven set at 70°C), these standards were injected into the HPLC. There was a shoulder on the internal standard peak in both samples.

All of the solutions used in the extraction process were made up fresh, and the extraction was performed again using standards of 250, 400, and 1000ng/mL. These standards continued to be characterized by the shoulder on the internal standard peak. A blood sample with neither cyclosporin A or C was extracted and injected to investigate the cause of the shoulder. The negative

control had peaks that appeared at the same time as the drug peaks in the extracted standards. In order to determine whether the interfering peaks originated from the extraction method or the instrument conditions, a new HPLC column was tested.

4.1c Waters HPLC with Jupiter column and Clean-Up® extraction

The Jupiter 5u C18 300A (250 x 4.6mm) column from Phenomenex was used with a mobile phase of 70:30 acetonitrile:water. Flow rates of 1.5mL/min and 2.0mL/min were both used with the oven at 70°C. The retention time of the peaks was early enough for a short run time, but they were too close together for adequate separation. The ratio of the mobile phase was altered in an attempt to achieve separation of the peaks. First a mobile phase of 80:20 acetonitrile:water was tried. The peaks, however, came out even sooner. A mobile phase of 60:40 acetonitrile:water was tried, with an improved separation. In order to decrease the run time, the oven temp was increased from 70°C to 80°C, with the flow remaining at 2.0mL/min. The peaks still separated nicely, and the retention time was less.

Using the Jupiter column and the Clean-Up® extraction method, the focus of the investigation shifted back to the problem of the interfering peaks. In order to determine if the interfering peak was related to the volume of internal standard added, two different concentrations and volumes were tested. The concentration of the first internal standard was 2,000ng/mL. The second internal standard was 20,000ng/mL. Two identical 2mL standards of cyclosporin A were prepared each with a concentration of 2,000ng/mL. These identical standards were then treated

with different volumes of the two internal standards, for a final concentration of 200ng/mL of cyclosporin C. The two specimens were extracted using the Clean-Up® extraction method and 10µL of each extracted standard was injected into the HPLC. A test solution was also injected in order to have a retention time comparison. The mobile phase used was 60:40 acetonitrile:water, with a flow rate of 2.0mL/min and the oven at 80°C.

The chromatography of both runs was messy with several peaks interfering with the internal standard peak. Measurements were taken using both area and peak height to determine the extraction efficiency. Unfortunately it was very low. The sample which had 200µl of internal standard added had a concentration of 214ng/mL using area, and 173ng/mL using peak heights. This is a recovery of approximately 10%. The sample which had 20µL of internal standard added had a value of 211ng/mL using area and 390ng/mL using peak height. This is a recovery of 10% or 20%.

All of the solutions used in the extraction process were prepared fresh, in an attempt to remove the interfering peaks from the chromatogram. This time a standard of 800ng/mL was prepared. Again, both internal standards were used. A non-extract was also prepared by adding internal standard to 2mL of an 800ng/mL cyclosporin A standard made up in methanol. This was dried down and reconstituted in 100µl of mobile phase. This was injected along with the two extracted samples.

The value of the non-extract was 786ng/mL by area and 717ng/mL by peak height. This is a recovery of 98% and 89% respectively. These extracted

samples had fewer interfering peaks, most likely as a result of using the newly prepared solutions in the extraction process.

The extracted sample containing the more concentrated internal standard had a calculated value of 570ng/mL by area and 445ng/mL by peak height. This is a recovery of 71% and 55% respectively.

4.1d Shimadzu HPLC with Synergi Max-RP column and Clean-Up® extraction

At this time, a new Shimadzu HPLC with auto-sampler was available for use. This new instrument was expected to dramatically decrease the amount of technician time needed for each run, as the instrument automatically makes each injection. In addition, the attached computer analyzes all of the data, automatically does the calibration, and calculates the results. Previously, these were all manual procedures. A new HPLC column was also put into use in order to observe run time, separation, and peak shape and size. The new column was a Synergi Max-RP C12 80A (150 x 4.6mm) column from Phenomenex.

A new mobile phase consisting of acetonitrile, and an ammonium sulfate buffer, methanol mixture was tried while maintaining the oven temperature at 80°C and the flow rate at 1.5mL/min. Several different ratios of the buffer to acetonitrile were tested to see which yielded the best combination of peak separation and run time. Changing the ratio of these components in the mobile phase is simplified on the new instrument. There are two pumps on the instrument. Once the ideal mobile phase is set, it can be prepared and both pumps used simultaneously so that wear and tear is equalized. While

experimenting with the ratio of the components in the mobile phase, however, the pumps are used separately. One pump is used for acetonitrile, and the other for the buffer mixture. Using the computer, the ratio of the flow from each pump could be altered. This saves time and materials by not having to make up batches of each different mobile phase simply to make one injection and dispose of it. Ratios of 50:50, 45:45, 40:60, 35:65, and 30:70 were all tried, with the 35:65 buffer:acetonitrile yielding the shortest run time while still adequately separating the peaks.

Now that the instrument conditions are satisfactory, the focus of the project shifts to optimizing the extraction method.

The Clean-Up® extraction method was chosen first because it had been successful earlier in this project. Several blood specimens were spiked to have a concentration of 800ng/mL and were extracted using the same method previously utilized. The resulting chromatograms were "very dirty" with many extraneous peaks interfering with the cyclosporin peaks. The extractions were repeated several times with fresh solutions, using both 1mL and 2mL sample sizes, with no significant changes.

Because this extraction had worked previously, it was anticipated that it would work with these instrument conditions. As this was not the case, other extractions were performed to determine if the problem originated with the extraction method, or the instrument conditions, such as HPLC column or mobile phase make up.

4.1e Shimadzu HPLC with Synergi Max-RP column and Oasis® extraction

Another solid phase extraction method was tested to determine its effectiveness. This method consists of using Water's Oasis® columns, which contain a copolymer with hydrophilic-lypophilic balance. The cyclosporin is extracted from blood with a precipitating reagent, applied to the column, washed, and then eluted with ethanol. The Oasis® columns are more expensive, but are still cost effective because they can be utilized several times prior to disposal. Blood was spiked to a value of 1,000ng/mL, extracted using the Oasis® method, and run on the HPLC. Unfortunately, there were also significant interfering peaks in this chromatogram. This extraction method is currently being used at the University of Washington Medical Center, with different instrumentation. Following the notes on their procedure, the extraction was repeated with specimens of 800ng/mL. Unfortunately, there were no noticeable improvements in the chromatograms.

Because the extraction procedure was both time consuming and labor intensive, the focus shifted from solid phase extractions to liquid-liquid phase extraction methods.

4.1f Shimadzu HPLC with Max-RP column and MTBE extraction

A liquid-liquid extraction method was chosen next. This method calls for acidifying 1mL of blood, adding MTBE, and then washing the ether with 95mM NaOH. Specimens were prepared at 80ng/mL and 800ng/mL of cyclosporin A. Internal standard, both cyclosporin C and D, was added to have a concentration of 800ng/mL each. A non-extract specimen was also prepared in methanol for

comparison. The chromatograms were much improved over any other extraction performed so far. The peaks were separated, with little interference by other peaks. Using cyclosporin C as the internal standard, and measuring by peak height, the recovery was 94% for the non-extract, and 68% for the extracted specimen.

4.1g Shimadzu HPLC with Max-RP column and diethyl ether extraction

These results were then compared to another liquid-liquid extraction, which utilizes diethyl ether. This method calls for acidifying the blood with HCl, mixing it with diethyl ether, evaporating the ether, reconstituting in HCl, washing with hexane, then adding NaOH and diethyl ether. The ether is evaporated and reconstituted in mobile phase. This is the method used at Mayo Laboratories. All of the volumes called for in the method were cut in half due to the available glassware and centrifuge capabilities. Standard of 800ng/mL were extracted and run on the HPLC along with a non-extract of the same value.

The extraction was repeated using MTBE in place of diethyl ether. MTBE is less volatile than diethyl ether, so the extraction takes longer, but it is much less dangerous to use and store.

Calculating with cyclosporin C as the internal standard, the result for the non-extract was 755ng/mL for a recovery of 94%. The extracted specimens had values of 711ng/mL for the extraction with diethyl ether, and 592ng/mL with MTBE, which means recoveries of 89% and 74% respectively. These values are misleading, however, because regardless of which solvent was used, the

cyclosporin A peak was either a double peak, or had a large shoulder on it, which would positively affect the values.

After comparing the results of all the different extraction methods, the first MTBE liquid-liquid extraction was chosen as the final method to be used. The solid phase extractions all had too many interferences, and too low of a recovery. This method was much easier and less time consuming than the second liquid-liquid extraction, with fewer interferences and similar recovery.

For a summary of the different instrument, column, and extraction combinations tested, and the procedure finally chosen, see Table 1.

HPLC	Column	Extraction
Waters HPLC	Bio-Rad column	Bio-Rad extraction
"	Prodigy column	Clean-Up extraction
"	Jupiter column	Clean-Up extraction
Shimadzu HPLC	Max-RP column	Clean-Up extraction
"	"	OASIS extraction
"	"	MTBE extraction
"	"	diethyl ether extraction
Final Combination:		
Shimadzu HPLC	Max-RP column	MTBE extraction

Table 1: Summary of instrument, column and extraction methods tested and the final combination chosen.

4.2 Improvement and Standardization

4.2a Improvement

Once the extraction method was finalized, small changes were made in order to decrease interferences, increase recovery, and remove any other problems with the method.

During the extraction, an emulsion would form while mixing the MTBE with the acidified specimen. Once the emulsion formed, it was extremely difficult to completely separate the two phases, even with continued centrifugation. Two changes in the extraction addressed this problem. First, the volume of MTBE used was increased from 6mL to 8mL. The increased volume removed most of the problem, but an emulsion still formed in some cases. The centrifuge tubes were almost completely full, so the second change was to use 15mL centrifuge tubes rather than 10mL tubes. The incorporation of both changes removed the emulsion problem.

Repeating the extraction again resulted in a peak interfering with the cyclosporin A peak. This interference was not evident in the non-extract, so it was concluded that the problem originated in the extraction process. Just prior to this, extraneous peaks had also been evident in GCMS extractions. After extensive testing of both chemicals and equipment, it was determined that the GCMS extraneous peaks were associated with the use of Eppendorf pipettes to dispense organic solvents.

The Eppendorfs were also being used in this extraction process. In order to clarify if the Eppendorf pipette caused the interference, mobile phase was transferred using the Eppendorf pipette, and then injected on the HPLC. The interfering peak was present. When, however, mobile phase was injected without using the Eppendorf, no peak appeared. The extraction was then repeated without using the Eppendorf pipette. The main interfering peak was no longer evident, but other smaller extraneous peaks still remained.

Other possible problem areas were considered in an attempt to completely remove the remaining interferences. The method used at Mayo called for rinsing all glassware utilized in the extraction in order to eliminate interferences. This procedure was performed prior to the next extraction. All of the tubes used in the extraction were rinsed first with 0.1M HCl and then with MTBE. The extraction was repeated, and the interfering peaks were greatly diminished.

Another variable affecting the recovery of cyclosporin was the solution used for reconstituting the specimen. Usually the mobile phase is used, but one of the methods called for using a solution of 3:1:1 76mM ammonium sulfate: acetonitrile:methanol, rather than mobile phase. Both the 3:1:1 solution and mobile phase were tried with several different injections. The 3:1:1 solution resulted in higher recovery and fewer extraneous peaks.

After the sample was reconstituted, it was rinsed with either hexane or heptane in order to remove a lipid peak. Heptane and hexane were both tested several times, with hexane producing a higher recovery with fewer interferences. Several injections were done without this wash step, in order to determine its significance. Without the wash step, many more interferences were evident, making it an important step in the extraction process.

Despite the improved recovery and the elimination of most interferences, an extraneous peak continued to elute very closely to cyclosporin A in the extracted samples. Although a definitive source was not identified, it was hypothesized to be a component of blood not removed during the extraction process. Changes were made to the flow rate and mobile phase ratio in order to separate the

peaks, and insure that no interference occurred. The mobile phase was changed from 35:65 buffer:acetonitrile to 37:63 buffer:acetonitrile. This caused the cyclosporin peaks to come out later, with the miscellaneous peak now between the cyclosporin C and cyclosporin A peaks, but still too close. The mobile phase ratio was then changed to 33:67, which caused the cyclosporin peaks to come out earlier and closer together. The flow rate was then lowered to 1.0mL/min in order to maintain adequate separation. The oven temperature remained at 80°C throughout. These changes caused the miscellaneous peak to be clearly separated from cyclosporin A, thereby eliminating the interference.

There are two different possible internal standards that can be utilized: cyclosporin C and cyclosporin D. Both were used throughout this project so that they could be compared with one another. There are advantages and disadvantages to each, including the recovery, separation from other peaks, and retention time. Cyclosporin C elutes first, which results in a shorter run time, but it is also closer to cyclosporin A, and separation is more difficult. Cyclosporin D elutes last, so the run time is longer, but separation is not a problem. Because of the difference in recovery, however, the obvious choice for internal standard for this project is cyclosporin C. Cyclosporin D had a much lower recovery than cyclosporin C. The peak shape was also better for cyclosporin C, with a taller, narrower peak.

4.2b Standardization

Following the criteria set forth in the "Tietz Textbook of Clinical Chemistry, Chapter 15, Selection and Evaluation of Methods" (4), the following steps were

performed to standardize the method. The therapeutic range for cyclosporin is 100-300ng/mL. A working internal standard solution was prepared at a concentration of 4,000ng/mL cyclosporin C and D. To each sample, 50µL of this internal standard was added, for a final concentration of 200ng/mL. A calibrator was prepared at a concentration of 200ng/mL. Bio-Rad® whole blood controls 1,2, and 3 were purchased and extracted and injected along with each run in order to establish ranges.

The standardization process included a linearity study to determine the range of the method. A limit of detection - limit of quantification study was performed at the low end of the linear range. Precision studies were performed, both with-in run, and run-to-run. A carry-over study was also performed. A second column of the same type was purchased, and specimens injected on both columns to insure the method was transferable from one column to the next. A comparison study was also performed with specimens that were sent to Mayo laboratory. Portions of the specimens were saved and sent to our laboratory where they were extracted and run, and the results compared to those of Mayo laboratory.

5.0 Final Results

Having identified the optimal instrumentation, extraction, and standardization, the final stage was to document the optimal performance of the analytical procedure.

In order to determine the analytical range, or linearity, of the method, specimens were prepared at concentrations of 25, 50, 100, 200, 400, 800, and 1,600ng/mL. These were extracted along with the calibrator and Bio-Rad® controls. After extraction, they were reconstituted in 150µL of the 3:1:1 solution mentioned previously, and 75µL were injected on the HPLC. The run proved to be linear between 50ng/mL and 800ng/mL.

The next step was to determine the limit of detection-limit of quantification. Ten negative specimens were run along with ten specimens spiked at 25ng/mL and ten at 50ng/mL. The coefficient of variation (CV) was calculated for all three values. The 50ng/mL group was the only one with a CV of less than 10. The results of these runs are listed in Table 2. The limit of detection was determined to be 50ng/mL.

The Student's t test was performed on the limit of detection-limit of quantification data in order to compare the three sets of data. This test compares two different groups of data in order to determine if they are statistically significant. This test shows again that 50ng/mL is the limit of detection, because this is the only group that is statistically significant from the other two.

negative	25 ng/mL	50 ng/ml
21.5	29.3	50.8
20.9	38.4	47.0
30.9	33.9	55.0
33.6	28.5	48.3
21.5	31.6	55.0
37.8	32.2	45.7
24.0	27.7	55.0
33.4	31.7	53.6
25.9	26.9	51.5
38.9	41.4	52.0
avg. = 28.8	avg. = 32.2	avg. = 51.4
sd = 6.57	sd = 4.44	sd = 3.25
cv= 22.8	cv = 13.7	cv = 6.32

Table 2: Limit of detection – limit of quantification

At a confidence level of 95%, and 9 degrees of freedom, the value of t is 3.25. When comparing the 25ng/mL and 50ng/mL sets, the value of t is calculated to equal 10.5. When comparing the negative and 50ng/mL sets, the value of t is calculated to equal 9.2. These values are greater than 3.25, which shows that the two groups are statistically different from one another. When comparing the negative and 25ng/mL sets, however, the value of t is 1.3. This shows that these two groups are not statistically significant from one another.

Portions of specimens, which were tested for cyclosporin by Mayo laboratory, were saved and tested using this new system. No names were involved in this study and the tubes were each labeled with a four-digit number and the result from Mayo. Thirty of these samples were extracted and run on the HPLC with data compared to the results sent by Mayo. The results are listed in Table 3. All

of the results calculated in this laboratory were within 10% of the results received from Mayo Laboratories.

patient number	expected result	result	comparison
6098	187	184	0.98
1234	487	476	0.98
5031	415	417	1.00
6071	123	131	1.07
6069	133	120	0.90
6009	<50	<50	1.00
1238	107	110	1.03
9009	144	154	1.07
2209	<50	<50	1.00
2024	141	149	1.06
5111	159	174	1.09
1009	204	197	0.97
2202	145	160	1.10
4020	160	161	1.01
2017	139	145	1.04
6010	151	158	1.05
5082	155	161	1.04
7007	134	135	1.01
9041	93	88	0.95
9006	213	199	0.93
7033	191	200	1.05
5039	192	186	0.97
1012	180	179	0.99
2061	74	77	1.04
1003	188	177	0.94
5010	150	147	0.98
1600	111	119	1.07
9001	155	169	1.09
1039	280	266	0.95
1784	115	110	0.96

Table 3: Results of thirty patient specimens, compared to results received from Mayo laboratory.

Precision was tested both with-in run and run-to-run. The with-in run precision was tested by spiking ten negative aliquots to a concentration of

300ng/mL. The specimens were extracted and run on the HPLC along with the calibrator and Bio-Rad® controls. The results are listed in Table 4. The coefficient of variation was calculated, and as it was under 10%, the precision results were acceptable.

300 ng/mL
274.4
317.3
275.7
318.4
319.5
323.8
304.8
330.1
294.8
313.0
average = 307.2
s.d. = 18.55
cv = 6.03

Table 4: With-in run precision results

Run-to-run precision was tested by spiking fifteen standards to a concentration of 100ng/mL, and fifteen at 300ng/mL. These specimens were extracted on different days and injected along with the calibrator and Bio-Rad® controls. The results of the analyses are shown in Table 5. The coefficient of variation was calculated, and was below 10 for both sets of values, so the run-to-run precision passed.

In order to determine if there is any carryover from one injection to the next at high concentrations, specimens were prepared and extracted at concentrations of 2,000ng/mL, 3,500ng/mL, and 5,000ng/mL. These specimens were injected

on the HPLC with a blank in between each one. There was no carryover to the blanks with any of the injections.

100 ng/mL	300 ng/mL
112.1	308.7
109.6	295.2
104.8	275.6
119.6	266.6
113.5	294.7
110.2	261.5
115.2	274.4
118.5	269.8
113.5	309.0
110.5	316.1
118.5	300.6
116.9	267.7
110.5	301.2
108.0	304.3
117.4	301.2
avg = 113.3	avg = 289.8
sd = 4.227	sd = 17.75
CV= 3.73	CV = 6.12

Table 5: Run-to-run precision results.

A new column of the same type was purchased from Phenomenex and conditioned on the HPLC. A calibrator and Bio-Rad® controls were extracted and run on each of the HPLC columns. This was done to insure that the same results would be achieved using either column. It also demonstrates that the extraction method and instrument conditions would not vary from one column to the other. The results were comparable regardless of which HPLC column was used.

6.0 Conclusions

The purpose of the research is to demonstrate a model for the development, improvement, and standardization of analytical methodologies for forensic toxicology laboratories. The first stage of research was designed to identify the optimal instrumentation parameters and extraction technique. First the HPLC was chosen as the best available instrument for the research; then the HPLC column, mobile phase and other instrument conditions and extraction method were optimized. This resulted in the selection of the Shimadzu HPLC, the Max-RP column from Phenomenex, a mobile phase of 35:65 acetonitrile:ammonium sulfate, a flow rate of 1.5mL/min, an oven temp at 80°C, and use of the MTBE extraction method.

Once this first stage of research was successfully completed, the stage was set for the improvement and standardization of the methodology.

The extraction technique was improved in several ways. A change in the volume of solvent resulted in the elimination of an emulsion, which had occurred during the extraction. Discontinuing use of the eppendorf pipettes eliminated some interference, and rinsing the glassware with acid and MTBE eliminated others. A slight change in the mobile phase ratio eliminated another interference. Optimization of the extraction then allowed standardization of the method to proceed.

The procedure was determined to be linear between 50 and 800ng/mL. The limit of detection – limit of quantification was proven to be 50ng/mL. The therapeutic range for cyclosporin is 100 to 300ng/mL, so this linear range and

lower limit is acceptable. The thirty specimens tested were all within ten percent of the results received from Mayo. Both the day-to-day precision and the within run precision tests had a CV of less than ten percent. The carryover limit is much higher than the upper limit of linearity, and should rarely, if ever, be an issue. The method was shown to work on a new column of the same type, which demonstrates the robustness of the method.

As a result of the combined stages of the research, this method is now optimized and standardized and ready for routine laboratory usage. Whenever a new drug comes into use, or if methods need to be standardized or sensitivity increased due to increased regulations, this research model can serve as a template for optimization and standardization. Similarly, in other forensic laboratories, by following the steps set forth in this project, an analytical method can be developed, improved, and/or standardized.

A generalized protocol for developing, optimizing, and standardizing a method is as follows:

1. Perform an inventory to determine what instrumentation and other resources are available. For instance, is appropriate instrumentation available, and if not, is there money to purchase new instrumentation, or are resources available to purchase standards and extraction columns?

2. Survey the literature and contact other laboratories to identify the latest methodologies and technologies. What instruments are being utilized, with what type of conditions, what type of extraction, and levels of sensitivity? Find out if there is a consensus, or if there are different opinions, and identify the reasons

behind them. For the purposes of this generalized protocol, it will be assumed that the favored instrument is the HPLC.

3. Select an analytical method. This includes the instrument, the conditions, such as column, mobile phase, flow rate, oven temp, etc. It also includes the type of extraction method. The most efficient technique is to choose an entire method that has been utilized with some level of effectiveness in another laboratory, as opposed to selecting parts of different methods and conducting extensive trial and error to piece them together. For example, choosing the extraction from one place, the HPLC column recommended in another, and the mobile phase from another would require extensive work, as shown in this project.

4. Analyze non-extracted standards on the instrument to optimize the instrument conditions before testing the extraction method. Utilizing multiple non-extract sample injections, each separate condition, such as flow rate or oven temperature are adjusted separately to ensure the optimized instrument conditions.

5. Identify the optimal extraction technique. Utilizing identical, and optimal, instrument conditions, the data obtained from the different extractions can be effectively compared to identify the most efficient extraction technique. The trend in laboratories now is to change from liquid-liquid extractions to solid phase extractions. There are several reasons for this. First, it limits the amount of solvents used in the extractions. Second, they tend to take less time, and efforts are now being made toward automation of the extractions. Third, they are a more powerful extraction technique, removing more impurities, and often have a

higher recovery. In this project, however, the research identified that a liquid phase extraction was more effective than three different solid phase extractions.

6. Optimize the entire method. The most effective procedure is to alter one criterion at a time, while leaving everything else constant. The method is shown to be completely optimized when by making any change, the results are, in some way, less effective.

7. Perform the standardization tests. This includes linearity, precision studies, limit of detection, limit of quantification, carryover, etc.

By following these general steps, a method can be identified, developed, optimized, and standardized for any type of drug, on any type of instrumentation.

7.0 Future Research

Future research can always be performed to improve methods by shortening run time, further improving separation, removing additional minor interferences, or improving recovery. This should be an on-going part of the laboratory process, along with reviewing methods to see what new procedures have been developed, and how the current procedures can be adapted.

A further study could be performed to test whether any other drugs co-elute with cyclosporin. Special attention should be given to those drugs that have similar physical characteristics to cyclosporin.

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