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Chiral Analyses of Ephedrines

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ABSTRACT

A majority of controlled substances are chiral compounds that have different toxicological effects in the body. Chiral molecules rotate plane-polarized light and have two or more forms known as enantiomers. Enantiomers share many chemical features including molecule weight and are difficult to separate. However, enantiomers interact differently with other chiral molecules which can be used for their separation. We plan to separate the two enantiomeric ephedrines using β-cyclodextrin column in gas chromatography-mass spectrometry (GC-MS). To achieve enatiomeric separation, a series of methods were developed through the modification of temperature and mobile phase parameters to separate the two ephedrine enantiomers. Ephedrine, a common decongestant, is derived from *Ephedra sinica* and a precursor for amphetamines and methamphetamines. After encountering issues with resolution and separation due to undesirable column and sample interactions, ephedrine salts were turned into free bases through acid-base extraction and acetylation of free-base ephedrines were performed. The final chromatography analysis of the ephedrine enantiomers resulted in the elution of two peaks present in each individual enantiomer chromatograph with slightly different mass fragmentation, indicating the presence of constitutional or conformational isomers. Based on the results it cannot be determined whether chiral separation can or cannot be achieved through the use of a β-cyclodextrin column in GC-MS using minimal derivatization procedures. At the same time, modification of the temperature programming and acetylation lead to better resolution in gas chromatography.

INTRODUCTION

Drug analysis and toxicology are important fields of chemistry because many drugs of interest have complex stereochemical properties, one of which is known as chirality, a feature of all asymmetrical organic molecules. Each chiral molecule has at least two unique forms that are non-superimposable mirror images of each other (like the left and the right hand), and each form is known as an enantiomer. Enantiomers share some of the same physical properties including boiling point, melting point, and densities but can be quite different in their functions and interactions in the human body. The toxicological effect of a drug in the body is highly affected by the stereochemistry of the compound because receptors bind differently with the compounds based on their shape. The different binding properties of enantiomers are caused by the interaction between the drug and its receptor. Each enantiomer has a different affinity, or likelihood of bonding based on energetic attraction for each receptor, and thus, has a different effect on the body (Mannschreck and Kiesswetter, 2005). An example of chirality in the body is the two forms of amino acids. 19 out of the 20 standard amino acids have chiral centers and each has a levorotatory and dextrorotatory enantiomer. Only the levorotatory amino acids are found in the proteins in the human body (Krause, et. al., 2000).

An important characteristic of enantiomers, also known as optical isomers, is that they rotate plane-polarized light differently. Enantiomers that rotate light to the left are known as levorotatory, or *(–)*, while enantiomers that rotate light to the right are known as dextrorotatory, or *(+)*. The stereochemistry of each molecule is determined by the configuration of the each chiral carbon present in the molecule, assigned based on the positions of the four different groups attached to the carbon atom. More specifically, the direction of the priority of the groups determines the configuration of the molecule. Priority is assigned based on the atomic number of

the constituent attached to the chiral carbon; the group with the highest atomic number is given the highest priority. The *(R)* configuration is given if the priority of the groups moves clockwise, while the *(S)* configuration is given if the priority of the groups moves counter-clockwise (Ingelse, 1997).

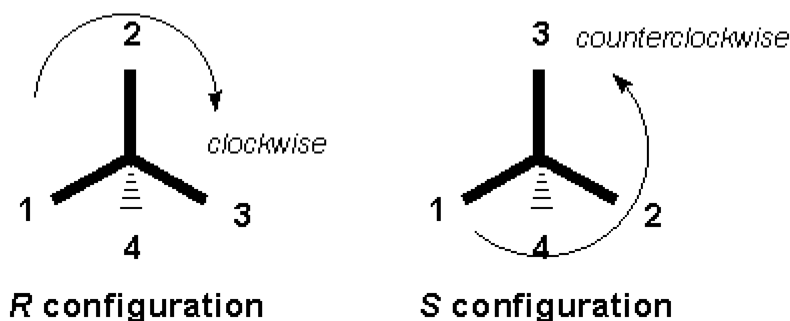


Figure 1. Assignment of the *(R)* and *(S)* Configurations. In the *(R)* configuration, the groups on the chiral carbon move from highest to lowest priority in a clockwise manner. In the *(S)* configuration, the groups on the chiral carbon move from highest to lowest priority in a counterclockwise manner.

Due to their difference in biological function, the separation and differentiation of enantiomers has become increasingly important. In the 1950s and 1960s, the use of a drug known as thalidomide was given to pregnant women to treat their morning sickness. However, this drug was a racemate, or an enantiomeric mixture, of N-phthalylglutamic acid imide and led to severe developmental defects in newborns due to the *(S)-(-)*-isomer. (Ikai and Okamoto, 2009) When enantiomers interact with other chiral molecules, they result in diastereomers because each enantiomer has different affinities to the chiral molecules. Diastereomers are stereoisomers that are not enantiomers and are not mirror images; they have partially different configurations of the chiral carbons. Diastereomers can be isolated more readily than enantiomers (Ingelse, 1997). Enantiomers have since been successfully isolated through chromatographic separation, specifically gas chromatography.

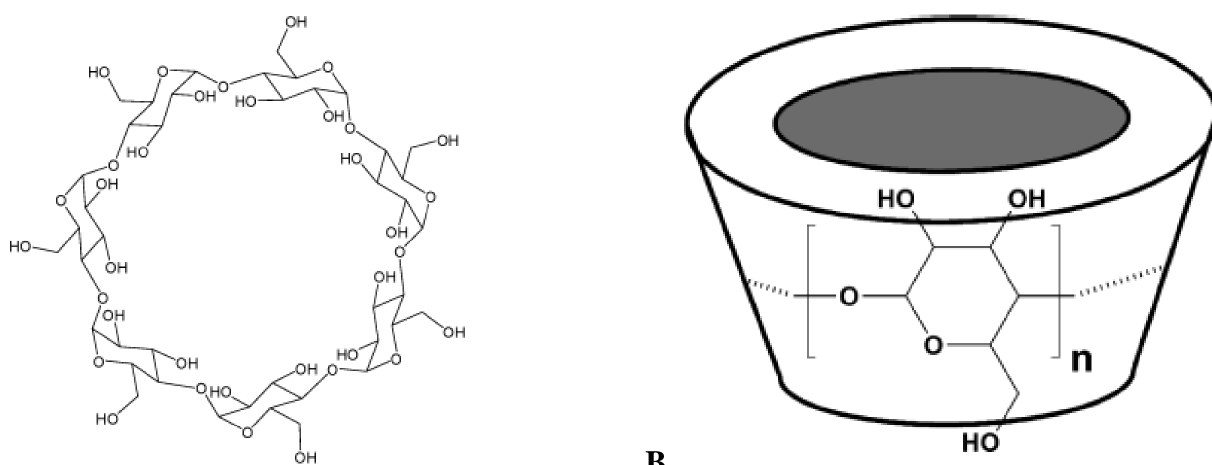
Chromatography is a method used for the physical separation of a mixture distributed throughout a stationary and mobile phase as different components are partitioned at different rates due to their affinities to the stationary phase. In gas chromatography, the mobile phase is known as a carrier gas, usually hydrogen, helium, or nitrogen gas. This phase is responsible for carrying the sample through the stationary phase that adsorbs the sample onto the column. The stationary phase is usually a liquid or nonvolatile compound immobilized to a solid support or column. Retention time, the time a compound is retained on the stationary phase, can be used to differentiate between different compounds found in a mixture. High affinity to the stationary phase results in a longer retention time, while low affinity to the stationary phase results in a shorter retention time. The retention time of a molecule is highly related to its physical properties. This means that similar molecules, like diastereomers, have similar retention times; while, enantiomers have exactly the same retention times. Separation of the sample components is displayed on the x-axis of the chromatograph representing the different retention times. The area of each peak on the chromatograph is proportional to the concentration of the substance.

Although sample separation is efficient with chromatograph, it is still difficult to know the exact identity of each component without a detector. Mass spectrometer is a detector used to determine the identity of an unknown compound. When the sample is ionized, it is separated into fragments of the original molecule, and those fragments are sorted by their mass-to-charge (m/z) ratio. Similar to the gas chromatographs, the height of each fragment peak is proportional to the concentration or amount found of each molecular weight.

The GC-MS is an instrument that carries out gas chromatography using a mass spectrometer. GC-MS is a useful analytical technique that is used to identify different components because of its sensitivity and effectiveness. In the GC-MS, a sample is injected into

the injection port with a syringe; where it is then vaporized to convert the sample into a gaseous phase. In capillary column gas chromatography, the injection chamber is separate from the column to ensure that only small amounts of the samples are loaded onto the column. As the sample passes through the column the retention times are dependent on the length of the column, the oven temperature, and the mobile phase flow rate (Fothergill, 1968).

Cyclodextrin is a chiral molecule that serves as a capillary stationary phase in high-performance liquid chromatography and gas chromatography. Cyclodextrin, also known as cycloamylose, is a cyclic chain of D-glucose joined together by α-1, 4-glycosidic linkages and is found to have a truncated cone shape. Cyclodextrins are synthesized from the enzymatic hydrolysis of starch. There are different types of cyclodextrin including alpha, beta, and gamma cyclodextrins that consist of 6, 7, and 8 D-glucose units, respectively. The hydroxyl groups of the cyclodextrin molecule are located on the outer surface; the primary hydroxyl groups are located towards the narrow portion, while the secondary hydroxyl groups are located towards the wider portion of the molecule (van de Manakker, et. al., 2009).



A. **B.**

Figure 2. From van de Manakker, et. al., 2009. (A) β-cyclodextrin Structure. β-cyclodextrin is produced by the microorganism *Bacillus macerans* responsible for the hydrolysis of starch. **(B) The Three-Dimensional Shape of a Cyclodextrin Molecule.** The wider portion of the molecule is the hydrophobic cavity with secondary hydroxyl groups, while the primary hydroxyl groups are in the narrow portion of cyclodextrin.

β-cyclodextrin can be used to coat a column, thereby giving it the ability to retain chiral compounds. Chiral columns are able to elute enantiomers at different retention times because the chiral stationary phase has a higher affinity to one enantiomer than with the other. The β-cyclodextrin column follows the "3-point interactions" principle which states that a chiral stationary phase interacts with each enantiomer differently, forming diastereomeric complexes through three or more interactions. These interactions include steric bulk hindrance, hydrogen bonding, dipole stacking, inclusion complexing, and π to π interactions (Keiluweit and Kleber, 2009). The hydrophobic portion of β-cyclodextrin allows nonpolar compounds to fit into the cavity through inclusion complex interaction. At the same time, hydrogen bonding between polar groups of compounds and the hydroxyl groups will occur; this will result in one enantiomer having a better fit in the β-cyclodextrin cavity than the other.

An example of a chiral molecule is ephedrine, which is a naturally derived amine that is found in *Ephedra* species, including *Ephedra sinica*, a common traditional Chinese herb Ma Huang. *Ephedra* is widely known for its medicinal properties in treating asthma, nasal congestion, and has been associated with weight loss as it serves as a diaphoretic drug (Valente, et. al., 2010). Although ephedrine is naturally derived and may be extracted from *Ephedra*, ephedrine may also be synthesized from propiophenone through bromination and amination or from glucose through fermentation and amination (Kurashima, et. al., 2004). Ephedrine has two chiral carbons and has two enantiomers, *(1R,2S)-(-)* and *(1S,2R)-(+)*-ephedrine, shown in Figure 2B, and two diastereomers, *(1S,2S)-(+)*-pseudoephedrine and *(1R,2R)-(-)*-pseudoephedrine, shown in Figure 4. *(1S,2S)-(+)*-pseudoephedrine and *(1R,2R)-(-)*-pseudoephedrine themselves are another pair of enantiomers. Enantiomers interact with an achiral stationary phase exactly the same way and thus are retained for the same amount of time on an achiral column, shown in

Figure 3A. Although the ephedrine and pseudoephedrine enantiomers are structurally similar and share several properties like gas chromatographic separation, the enantiomers are converted into two different forms of amphetamine and methamphetamine. The *(1R,2S)-(-)*-ephedrine and *(1S,2S)-(+)*-pseudoephedrine are highly converted into the psychoactive form of methamphetamine (Drake, et. al., 2011). As a result, the United States Drug Enforcement Administration has listed the enantiomers of ephedrine and pseudoephedrine as List 1 chemicals.

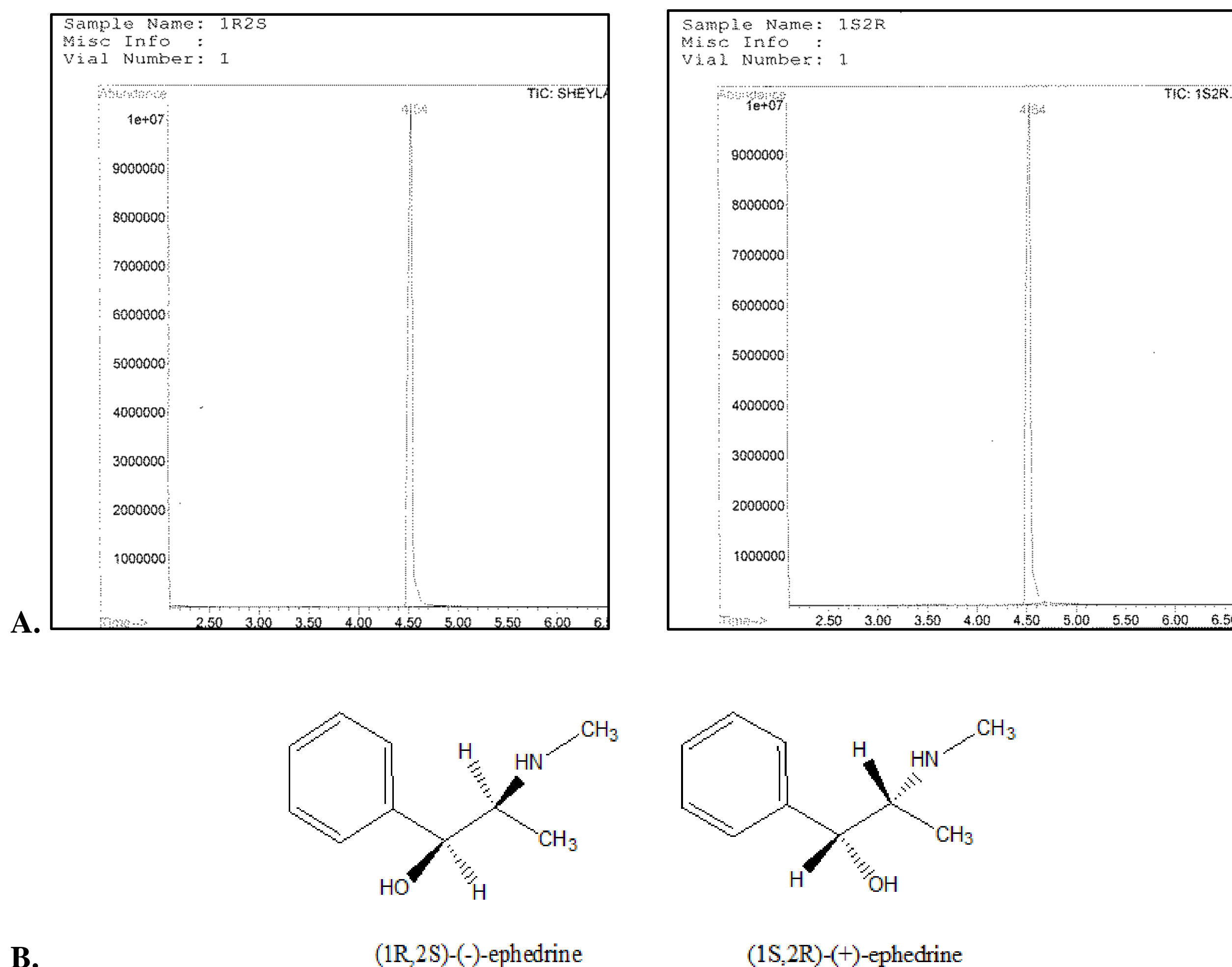


Figure 3. (A) Chromatographs of *(1R,2S)-(-)* and *(1S,2R)-(+)*-Ephedrine. These two chromatographs belong to *(1R,2S)-(-)* and *(1S,2R)-(+)*-ephedrine and show that on an achiral column the two enantiomers are eluted at the same time, having a retention time of 4.54 minutes. **(B) The Structures of the Two Ephedrine Enantiomers.**

There are several tests that are used in the identification of drug agents. However, there is a lack of efficient and economic identification methods of drug agents and their enantiomers. The importance of differentiation of drug enantiomers can be observed in the difference between *(S)* and *(R)*-methamphetamine. *(S)*-methamphetamine is the compound that is known as "meth," a narcotic with psychoactive properties; while *(R)*-methamphetamine is not psychoactive and is an over-the-counter drug found in Nyquil and Vicks vapor inhaler (Paul, et. al., 2004).

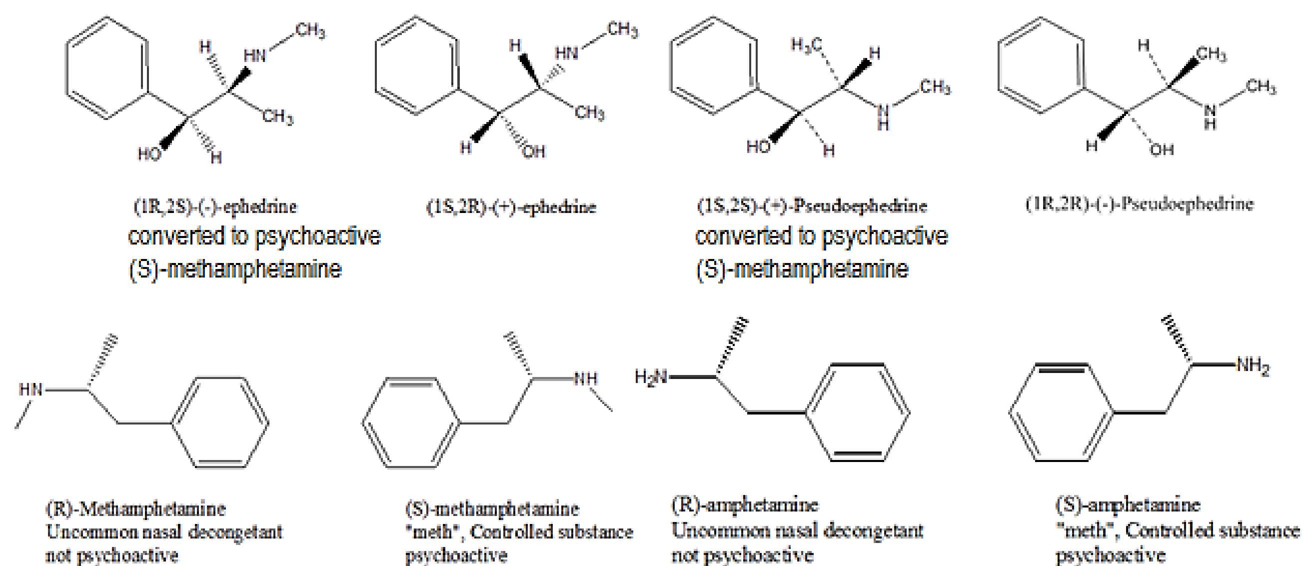


Figure 4. Structures of Controlled Substances and their Enantiomers. Ephedrine has two enantiomers and two diastereomers known as pseudoephedrine. These compounds are used to manufacture structurally similar psychoactive amphetamine and methamphetamine. The psychoactive forms of "meth" are the *(S)*-amphetamine and methamphetamine. Precursors for the psychoactive forms of "meth" are *(1R,2S)-(-)*-ephedrine and *(1S,2S)-(+)*-pseudoephedrine.

As drug detection and toxicity are growing fields in forensic science, important strides have been made in these areas. Enantiomeric analysis and chiral separation of ephedrine, pseudoephedrine, amphetamine, and methamphetamine has been previously documented. Amphetamine and methamphetamine have been isolated from urine and enantiomerically isolated through derivatization with Marfey's reagent or 1-fluoro-2,4-dinitrophenyl-5-I-aniline amide. This resulted in the production of diastereomers, which were then separated on a reverse-

phase high performance liquid chromatography (HPLC) column (Foster, et. al., 1998). Ephedrine has been isolated from *Ephedra sinica* and examined through first dimension β-cyclodextrin column and second dimension polar polyethylene glycol column gas chromatography-flame ionization detector (GC-FID). The second dimension GC-FID column eliminated the need for derivatization, matrix interference, and enhances detection sensitivity (Wang, et. al., 2006).

Despite the work already published, there is still a lack of efficient and economic chiral separation techniques without sample preparation using a chiral derivatization agent. In our labs, enantiomeric separation of ephedrine has been successful with the use of a chiral derivatization agent, L-TPC ((L)-N-trifluoroacetyl prolyl chloride). The acyl functional group of the bulky L-TPC reagent reacts solely with the amine functional group of the ephedrine and not the hydroxyl functional group due to steric hindrance, as seen in Figure 5. When the two ephedrine enantiomers react with L-TPC, they form diastereomers. When analyzed using an achiral polysiloxane GC column, commonly known as HP-5, the two diastereomers had different retention times, as seen in Figure 6. The problem with chiral derivatization is that this technique is time-consuming and the reagents are expensive and readily oxidized after one use, making it less than ideal for rapid, inexpensive drug detection.

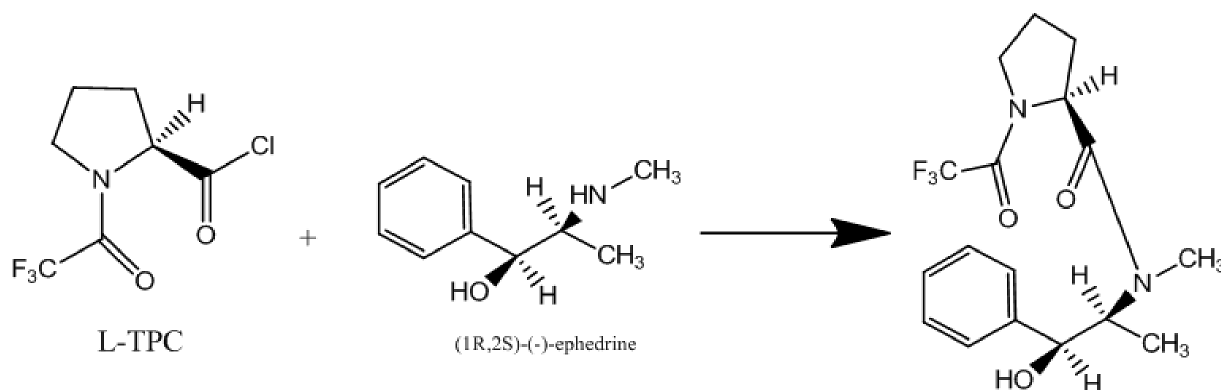


Figure 5. Mechanism of L-TPC and *(1R,2S)-(-)*-Ephedrine. The chiral derivatization agent, L-TPC reacts with each ephedrine enantiomer to form diastereomers. Reaction occurs between the nucleophilic amine group of the ephedrine and the electrophilic acyl group of the L-TPC.

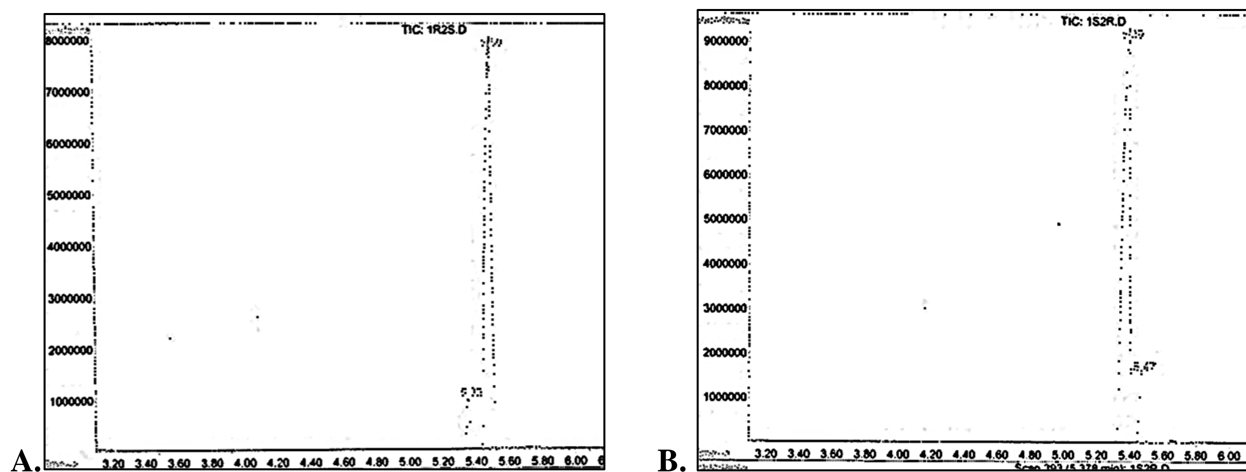


Figure 6. Chromatographs of the Ephedrine Enantiomers & L-TPC Diastereomers. These two chromatographs belong to the diastereomers formed with L-TPC and A.*(1R,2S)-(-)* and B.*(1S,2R)-(+)*-ephedrine on an HP-5 column. The retention time of the two diastereomers were slightly different.

OBJECTIVE

The goal of this research is the recognition and separation of ephedrine enantiomers, which are drug amine precursor, through the use of a chiral β-cyclodextrin GC column. This will require the use of different organic chemistry techniques and the development of several GC-MS methods.

MATERIALS & METHODS

Materials.

(1R,2S)-(-)-Ephedrine hydrochloride (99%) and *(1S,2R)-(+)*-Ephedrine hydrochloride (99%) were purchased from Sigma-Aldrich. Acetic Anhydride was purchased from Acros Organics.

Equipment

A Hewlett-Packard Agilent 6890 Gas Chromatograph with a 5973 Mass Selective Detector was used. The GC column was a RESTEK RT-bDEXsp chiral beta cyclodextrin column with a maximum temperature of 230°C, nominal length of 30.0 m, nominal diameter of 250 µm, and a nominal film thickness of 0.25 µm. In addition, the BUCHI Rotavapor R-3000 was used.

Free-Base Extraction

(1R,2S)-(-)-ephedrine hydrochloride was dissolved in 6M sodium hydroxide and brought to a pH of 12. Using dichloromethane the *(1R,2S)-(-)*-ephedrine free base form was extracted into the organic layer. The *(1R,2S)-(-)*-ephedrine was then dried with sodium sulfate and the dichloromethane solvent was removed using a rotary evaporator. The extraction procedures were repeated with *(1S,2R)-(+)*-ephedrine hydrochloride and both enantiomers were stored at 2-7°C.

Derivatization with Acetic Anhydride

To achieve acetylation of ephedrine, a protocol was adapted from Huffine and Canfield, 1992. An excess amount of acetic anhydride was added to the free base form of *(1R,2S)-(-)*-ephedrine and stirred for two hours. The mixture was flushed with nitrogen gas and sealed with a rubber balloon stopper. Excess nitrogen gas was added from a balloon attached to a syringe and was injected into the balloon stopper, seen in Figure 7. The mixture was heated while stirring to 70°C for 30 minutes. After it was cooled to room temperature, the *(1R,2S)-(-)*-ephedrine derivative was extracted using 2:1 mixture of dichloromethane and water. The unreacted acetic anhydride was converted into acetic acid in the presence of water and the *(1R,2S)-(-)*-ephedrine derivative was obtained in the organic layer. The organic layer was dried with sodium sulfate and the dichloromethane solvent was removed using a rotary evaporator. The derivatization procedures were repeated with the free base form of *(1S,2R)-(+)*-ephedrine and both acetylated enantiomers were stored at 2-7°C.

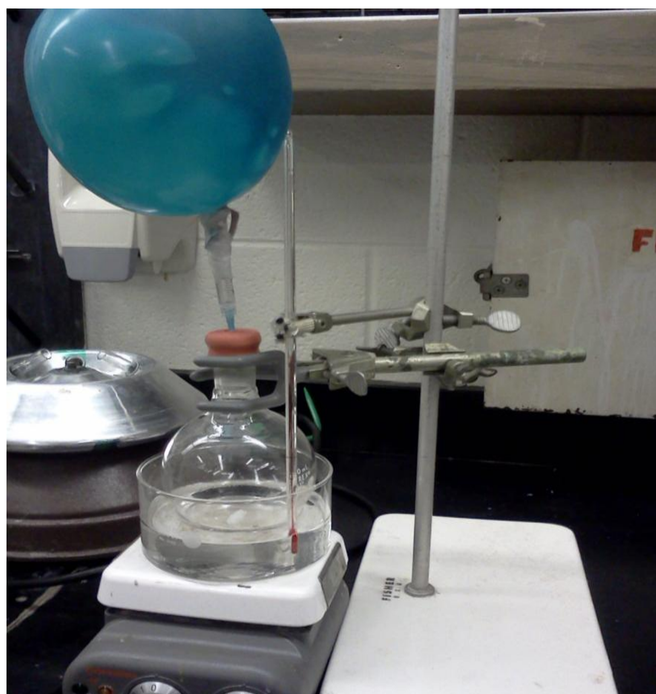


Figure 7. Acetic Anhydride Derivatization Apparatus.

The two enantiomers were tested using GC-MS to determine if the ephedrine derivatization were successful. The acetylated *(1R,2S)-(-)*-ephedrine was then combined with the aqueous layer. Sodium bicarbonate was added to the mixture and brought to a pH of 7. Using dichloromethane the acetylated *(1R,2S)-(-)*-ephedrine was extracted and obtained in the organic layer. The acetylated *(1R,2S)-(-)*-ephedrine was dried with sodium sulfate and the dichloromethane solvent was removed under a rotary evaporator. The extraction procedures were repeated with acetylated *(1S,2R)-(+)*-ephedrine and both enantiomers were stored at 2-7°C.

GC-MS Method Parameters

DAICHIRAL.

Start Temperature: 65°C Hold: 1 minute Temperature Rate: 15°C/minute

End Temperature: 225°C Hold: 4 minutes

The injector and detector temperature were 230°C and the injection volume was 1 μL. The initial flow rate of the helium gas was 1.5 mL/minute, the nominal initial pressure was 14.09 psi, and the average velocity was 46 cm/second.

LVCHIRAL.

Start Temperature: 100°C Hold: 1 minute Temperature Rate: 5°C/minute

Start Temperature: 140°C Hold: 9 minutes Temperature Rate: 10°C/minute

End Temperature: 175°C Hold: 4 minutes

The injector temperature was 200°C and detector temperature was 230°C, while the injection volume was 1 μL. The initial flow rate of the helium gas was 1.6 mL/minute, the nominal initial pressure was 17.59 psi, and the average velocity was 48 cm/second. (Adapted from Drake, et. al., 2011)

LVCHIRL2.

Start Temperature: 120°C Hold: 1 minute Temperature Rate: 1.5°C/minute

End Temperature: 175°C Hold: 3 minutes

The injector temperature was 120°C and detector temperature was 220°C, while the injection volume was 1 µL. The initial flow rate of the helium gas was 0.4 mL/minute, the nominal initial pressure was 2.80 psi, and the average velocity was 25 cm/second.

LVCHIRL3.

Start Temperature: 120°C Hold: 1 minute Temperature Rate: 1.5°C/minute

End Temperature: 175°C Hold: 3 minutes

The injector temperature was 120°C and detector temperature was 220°C, while the injection volume was 1 µL. The initial flow rate of the helium gas was 1.5 mL/minute, the nominal initial pressure was 17.97 psi, and the average velocity was 47 cm/second.

LVCHIRL4.

Start Temperature: 70°C Hold: 1 minute Temperature Rate: 10°C/minute

Start Temperature: 100°C Hold: 20 minutes Temperature Rate: 1.5°C/minute

End Temperature: 160°C Hold: 10 minutes

The injector temperature was 200°C and detector temperature was 220°C, while the injection volume was 2 µL. The initial flow rate of the helium gas was 1.2 mL/minute, the nominal initial pressure was 11.05 psi, and the average velocity was 40 cm/second. (Adapted from Wang, et. al., 2006)

LVCHIRL5.

Start Temperature: 70°C Hold: 0 minutes Temperature Rate: 10°C/minute

Start Temperature: 125°C Hold: 1 minute Temperature Rate: 0.5°C/minute

End Temperature: 145°C Hold: 5 minutes

The injector temperature was 200°C and detector temperature was 220°C, while the injection volume was 2 μL. The initial flow rate of the helium gas was 1.2 mL/minute, the nominal initial pressure was 11.05 psi, and the average velocity was 40 cm/second.

LVCHIRL6.

Start Temperature: 70°C Hold: 0 minutes Temperature Rate: 10°C/minute

Start Temperature: 125°C Hold: 1 minute Temperature Rate: 1°C/minute

End Temperature: 145°C Hold: 5 minutes

The injector temperature was 200°C and detector temperature was 220°C, while the injection volume was 2 μL. The initial flow rate of the helium gas was 1.2 mL/minute, the nominal initial pressure was 11.05 psi, and the average velocity was 40 cm/second.

LVCHIRL7.

Start Temperature: 70°C Hold: 0 minutes Temperature Rate: 10°C/minute

Start Temperature: 125°C Hold: 1 minute Temperature Rate: 2°C/minute

End Temperature: 145°C Hold: 5 minutes

The injector and detector temperature was 220°C, while the injection volume was 2 μL. The initial flow rate of the helium gas was 1.2 mL/minute, the nominal initial pressure was 11.04 psi, and the average velocity was 40 cm/second.

LVCHIRL8.

Start Temperature: 70°C Hold: 0 minutes Temperature Rate: 15°C/minute

End Temperature: 220°C

The injector and detector temperature was 220°C, while the injection volume was 2 μL. The initial flow rate of the helium gas was 1.2 mL/minute, the nominal initial pressure was 11.04 psi, and the average velocity was 40 cm/second.

LVCHIRL9.

Start Temperature: 60°C   Hold: 0 minutes   Temperature Rate: 2°C/minute

End Temperature: 220°C

The injector and detector temperature was 220°C, while the injection volume was 2 μL. The initial flow rate of the helium gas was 1.2 mL/minute, the nominal initial pressure was 10.41 psi, and the average velocity was 40 cm/second (Adapted from Tabacchi, et. al., 1997).

LVCHRL10.

Start Temperature: 100°C   Hold: 1 minute   Temperature Rate: 10°C/minute

Start Temperature: 140°C   Hold: 20 minutes   Temperature Rate: 1.5°C/minute

End Temperature: 180°C   Hold: 10 minutes

The injector temperature was 200°C and detector temperature was 220°C, while the injection volume was 2 μL. The initial flow rate of the helium gas was 1.2 mL/minute, the nominal initial pressure was 11.05 psi, and the average velocity was 40 cm/second.

RESULTS

To investigate the chiral separation of *(1R,2S)-(-)* and *(1S,2R)-(+)*-ephedrine, the use of a β-cyclodextrin column for GC-MS was employed. First, the chiral separation was analyzed using the DAICHIRL method with a 1:1 mixture of *(1R,2S)-(-)*-ephedrine hydrochloride and *(1S,2R)-(+)*-ephedrine hydrochloride. The DAICHIRL method was developed to determine if the column will recognize ephedrine and evaluate any initial chiral separation. This method was designed with a starting temperature of 65°C was 5°C above the column minimum and an end temperature of 225°C was 5°C below the column maximum at a constant rate of 15°C/minute. Two peaks were obtained along with a benzaldehyde peak at 5.84 minutes due to the methanol solvent column interactions during this method shown in Figure 8A. Interestingly enough, the mass spectra of the two peaks were not similar (Figures 8B and 8C) and after analysis of each enantiomer resulted in similar chromatographs, it was determined that there was no chiral separation.

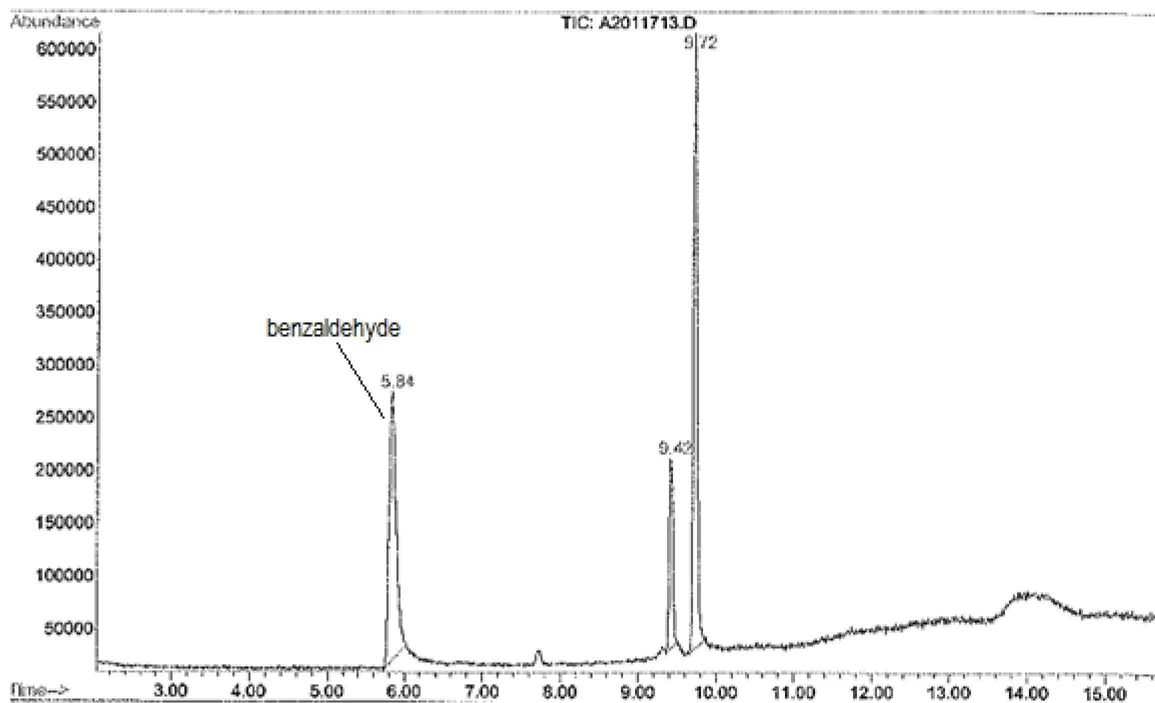


Figure 8A. Chromatograph of *(-)* and *(+)*-ephedrine hydrochloride using DAICHIRL.

There were three major peaks, one at 5.84 minutes that was characteristic of benzaldehyde, and the other two peaks at 9.42 and 9.72 minutes.

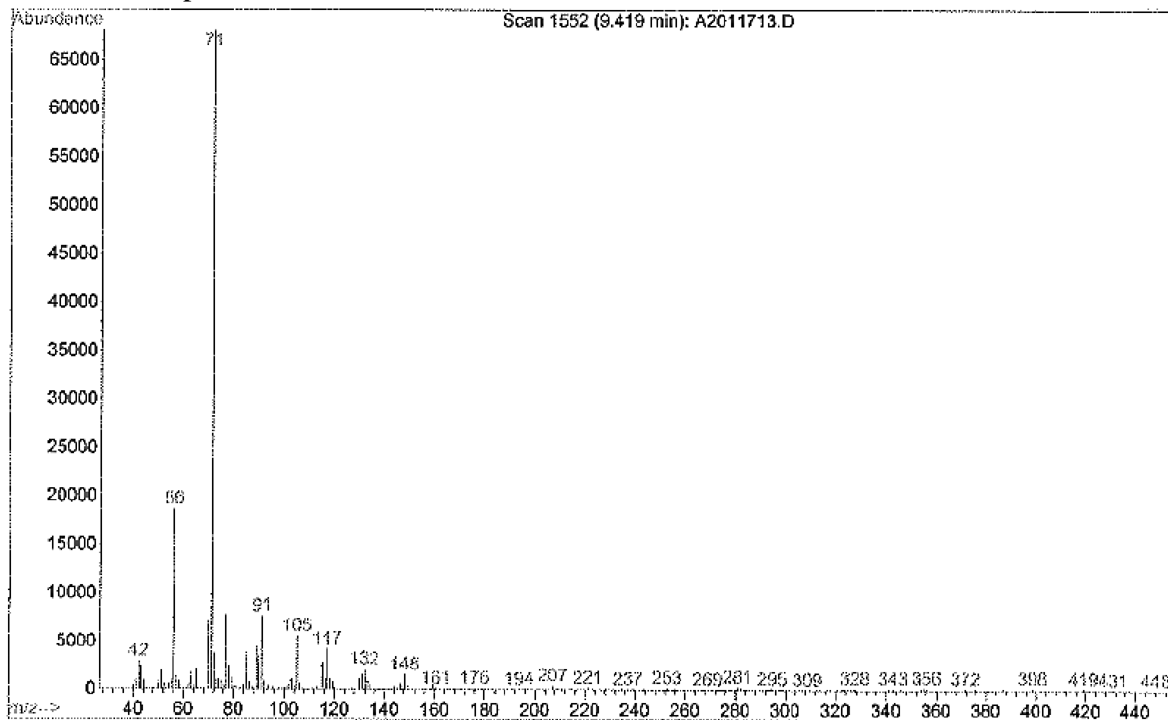


Figure 8B. Mass Spectra of the Peak with a Retention Time of 9.42 Minutes.

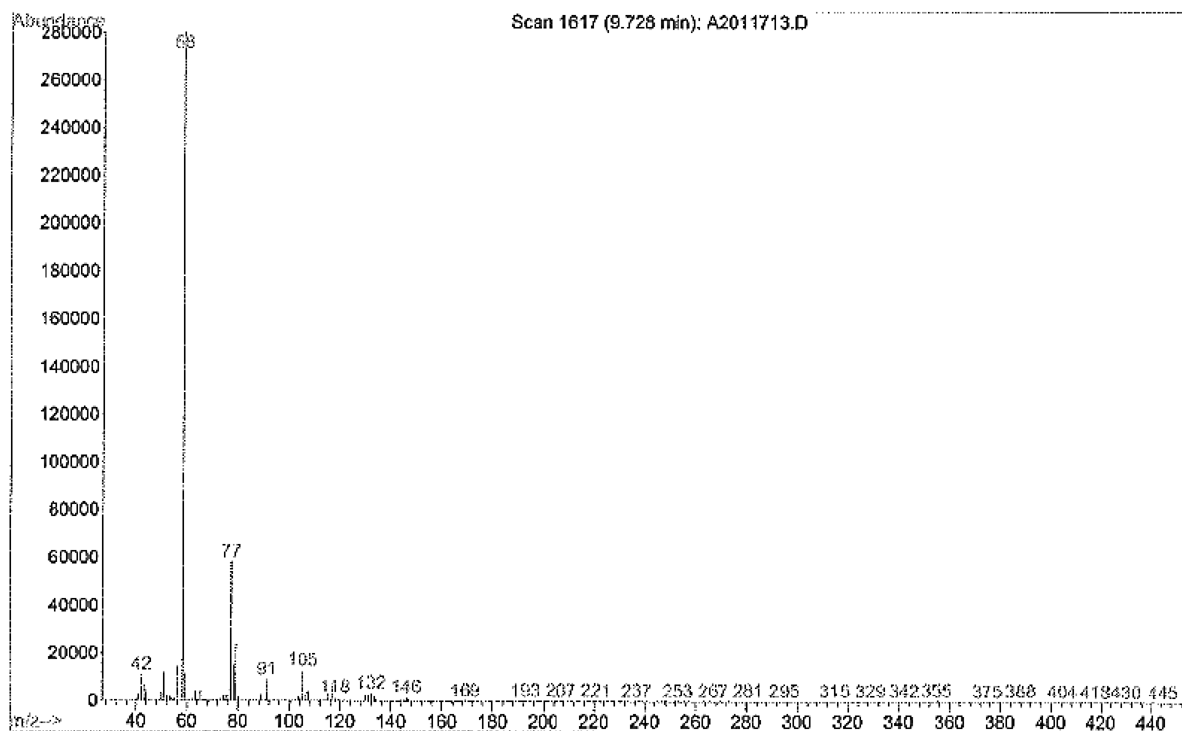


Figure 8C. Mass Spectra of the Peak with a Retention Time of 9.72 Minutes.

Based on the retention time of the peaks, LVCHIRAL was developed. The LVCHIRAL method was adapted from Drake who used a γ-cyclodextrin GC-MS column in 2011 (Drake, et. al., 2011). This method was designed at a higher starting temperature of 100°C at two slower temperature rates (see methods section) and a slightly higher helium flow rate. LVCHIRL2 and LVCHIRL3 were developed from LVCHIRAL reducing the temperature rate and alternating between lower and high helium flow rates. These methods were developed to enhance chiral separation by slowing the temperature rate which would in turn lead to better efficiency. Following analysis of ephedrine with LVCHIRAL, LVCHIRL2, and LVCHIRL3 all of which resulted in empty chromatographs, the LVCHIRL4 method was developed. The LVCHIRL4 temperature programming was adapted from Wang who used a β-cyclodextrin GC-FID column and achieved enantiomeric separation in 2006. The chiral separation of the two ephedrine hydrochloride enantiomers was analyzed using the LVCHIRL4 method. Figure 9A shows that there is some chiral separation of the two enantiomers when the LVCHIRL4 method was used. When each enantiomer was analyzed individually using the same method, the enantiomers had slightly different retention times but were broad and unresolved peaks, as seen in Figures 9B and 9C.

To resolve these issues, an extraction was performed for each ephedrine hydrochloride enantiomer to obtain the free base form of each compound. After analysis with the LVCHIRL4 method determined that there was no chiral separation, the LVCHIRL5, LVCHIRL6, and LVCHIRL7 methods were development. LVCHIRL5, LVCHIRL6, and LVCHIRL7 had similar temperature programming to determine if better resolution with slight modifications to the temperature rate. LVCHIRL8 was developed to flush out the column of all compounds to minimize the run time of LVCHIRL5, LVCHIRL6, and LVCHIRL7. With the LVCHIRL7

method, there was the beginning of chiral separation seen in Figure 10. LVCHIRL9 was adapted from the Restek chiral chromatography guide but was unsuccessful in chiral separation (Tabacchi, et. al., 1997).

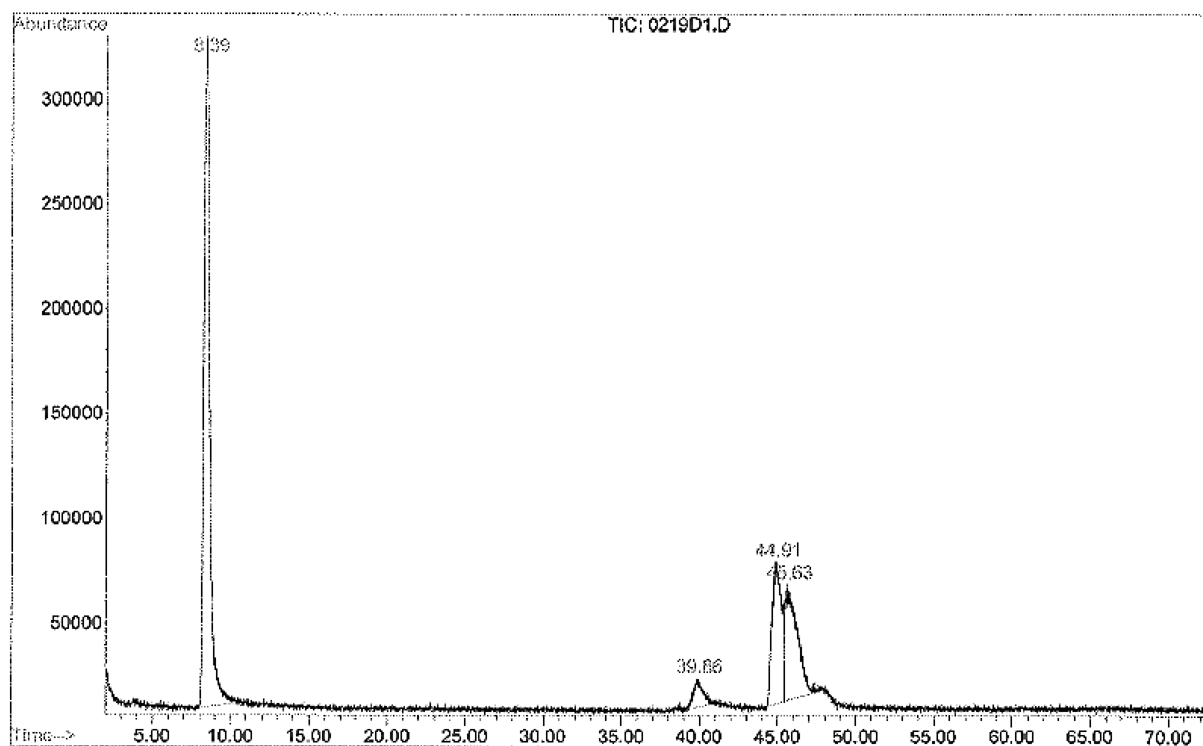


Figure 9A. Chromatograph of *(-)* and *(+)*-ephedrine hydrochloride using LVCHIRL4. There is a major solvent peak at 8.38 minutes and two major unresolved peaks at 44.91 minutes, and at 45.63 minutes.

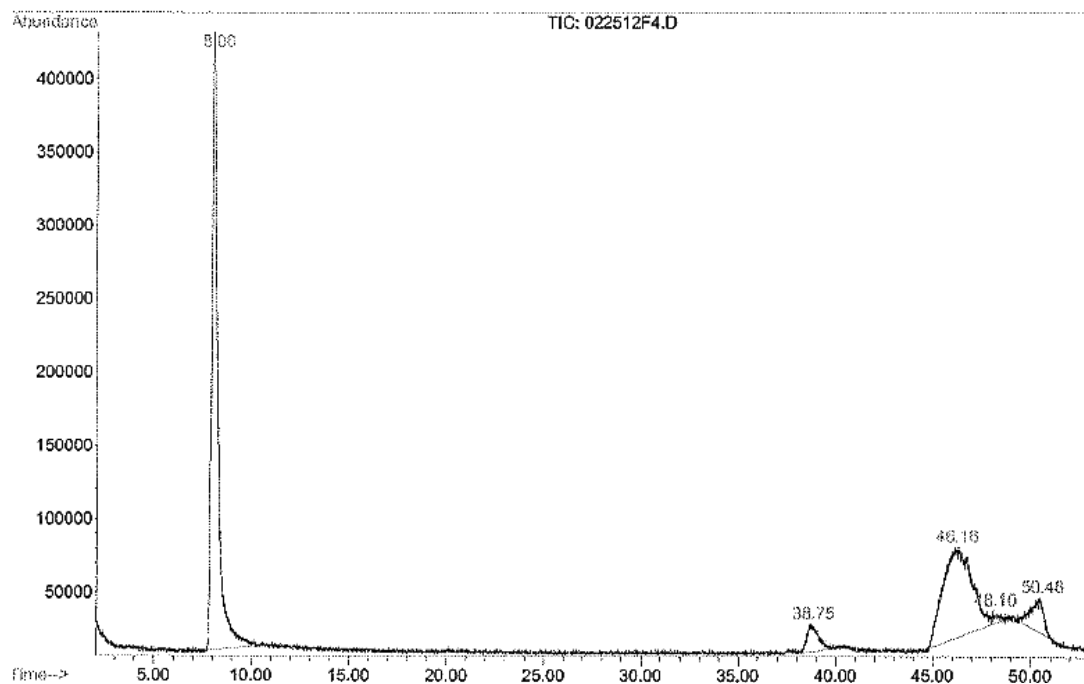


Figure 9B. Chromatograph of *(1R,2S)-(-)*-ephedrine hydrochloride using LVCHIRL4. There is a major solvent peak at 8.38 minutes as in the mixture and one broad peak at 46.16 minutes with tailing until 50.48 minutes.

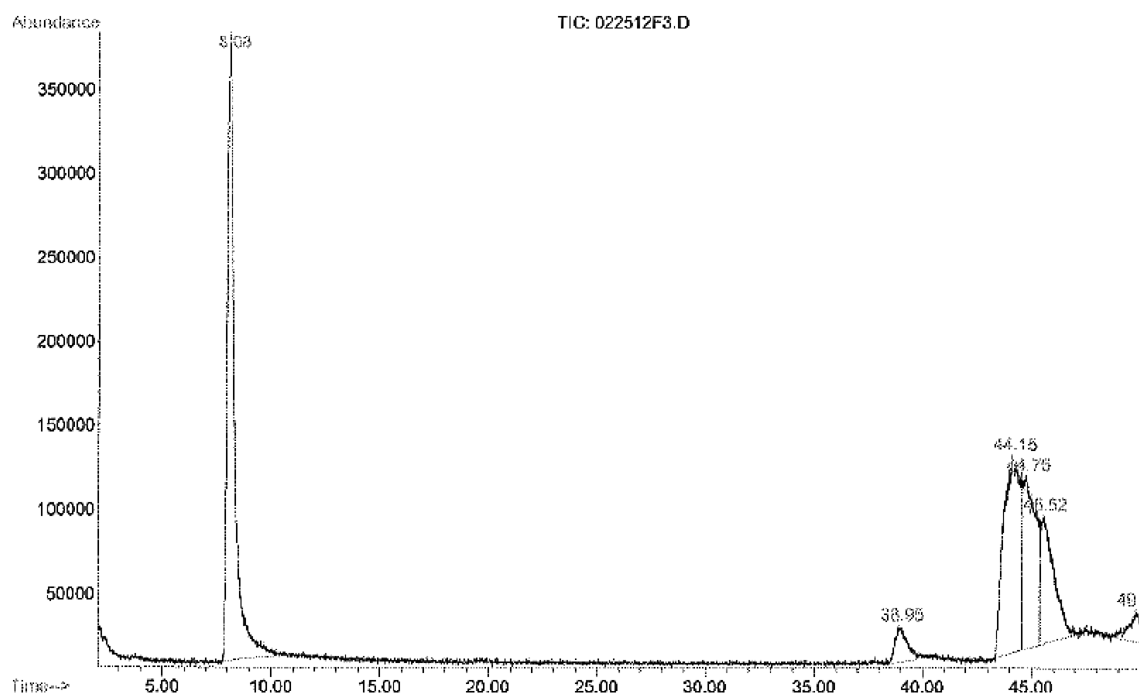


Figure 9C. Chromatograph of *(1S,2R)-(+)*-ephedrine hydrochloride using LVCHIRL4. There is a major solvent peak at 8.38 minutes as in the other two chromatographs and one broad peak at 44.15 until 45.52 minutes with tailing until 49.8 minutes.

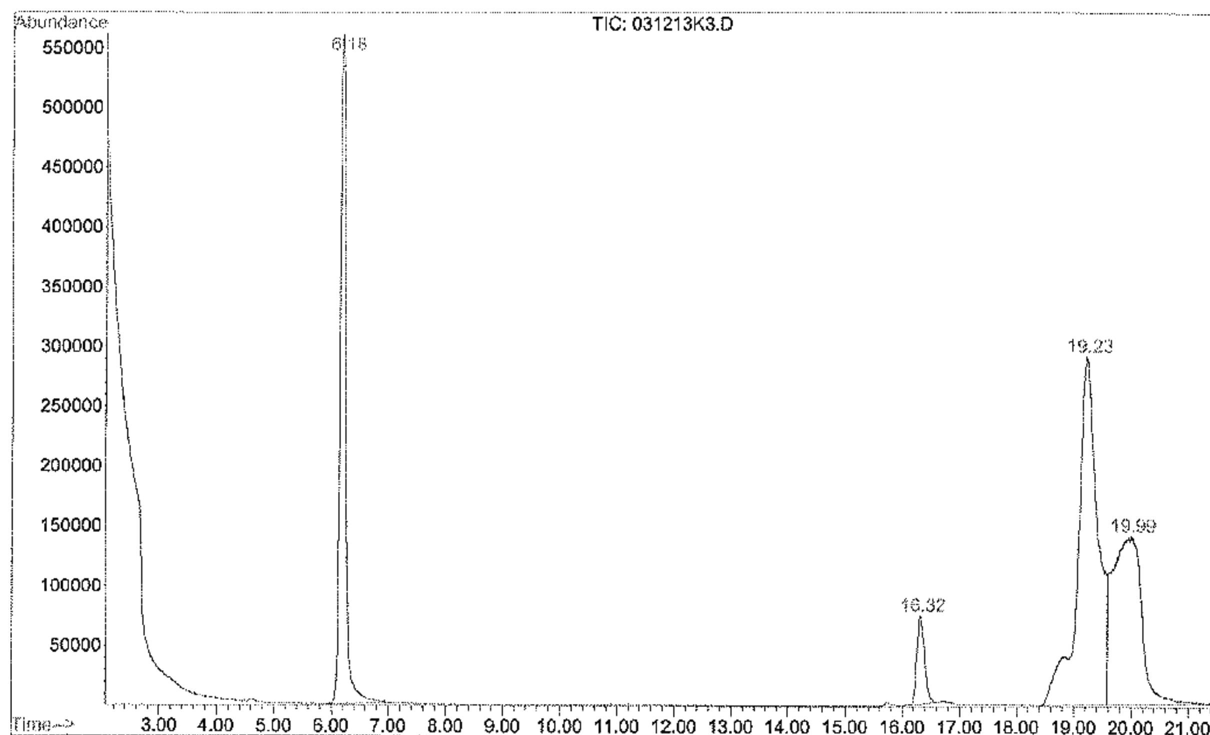


Figure 10. Chromatograph of free base *(-)* and *(+)*-ephedrine using LVCHIRL7. There is a major solvent peak and a benzaldehyde peak at 6.18 minutes due to the methylene chloride solvent column interactions during this method. The other two major peaks were unresolved at 19.23 and 19.99 minutes.

To achieve better separation, an acetylation derivatization was performed on each of the free base forms of ephedrine. Acetylation limits the hydrogen bonding between hydrophilic groups of ephedrine and hydroxyl groups of β-cyclodextrin which would result in shorter retention times and better peak resolution. The chiral separation of the two acetylated forms of ephedrine mixture was analyzed using the LVCHRL10 method. LVCHRL10 was developed from LVCHIRL4, one of the more efficient methods used throughout this project. The temperature rate was kept constant while the temperature range was changed to resolve issues of broad peaks. Two major peaks were obtained along with a solvent peak shown in Figure 11A. The mass spectra for the two peaks, Figures 11B and 11C, have very similar fragmentation indicating that the two peaks are conformational or constitutional isomers. The two peaks that are present in the acetylated ephedrine mixture, Figure 11A, are both present in the

chromatographs of the individual acetylated ephedrine mixture shown in Figures 12A and 12B, indicating that chiral separation has yet to be achieved.

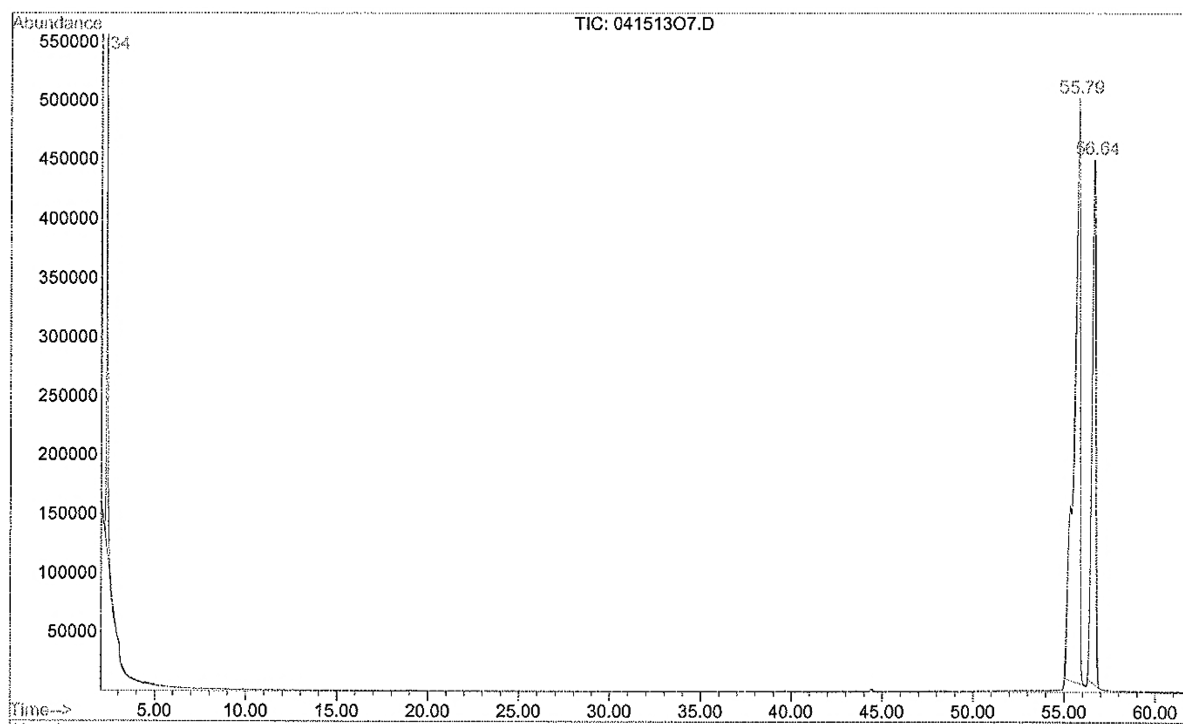


Figure 11A. Chromatograph of *(-)* and *(+)*-acetylated ephedrine using LVCHRL10. There is a major solvent peak along with two major peaks, one at 55.79 minutes and the other at 56.64 minutes.

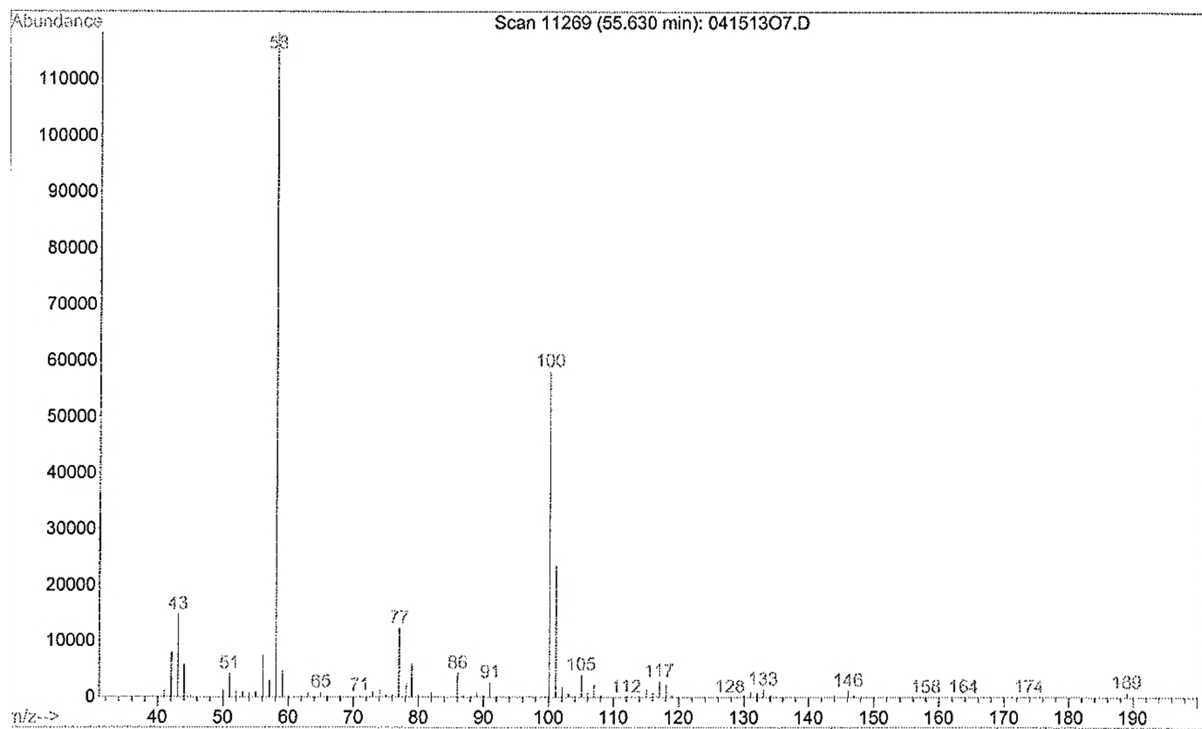


Figure 11B. Mass spectra of the peak with a retention time of 55.79 minutes.

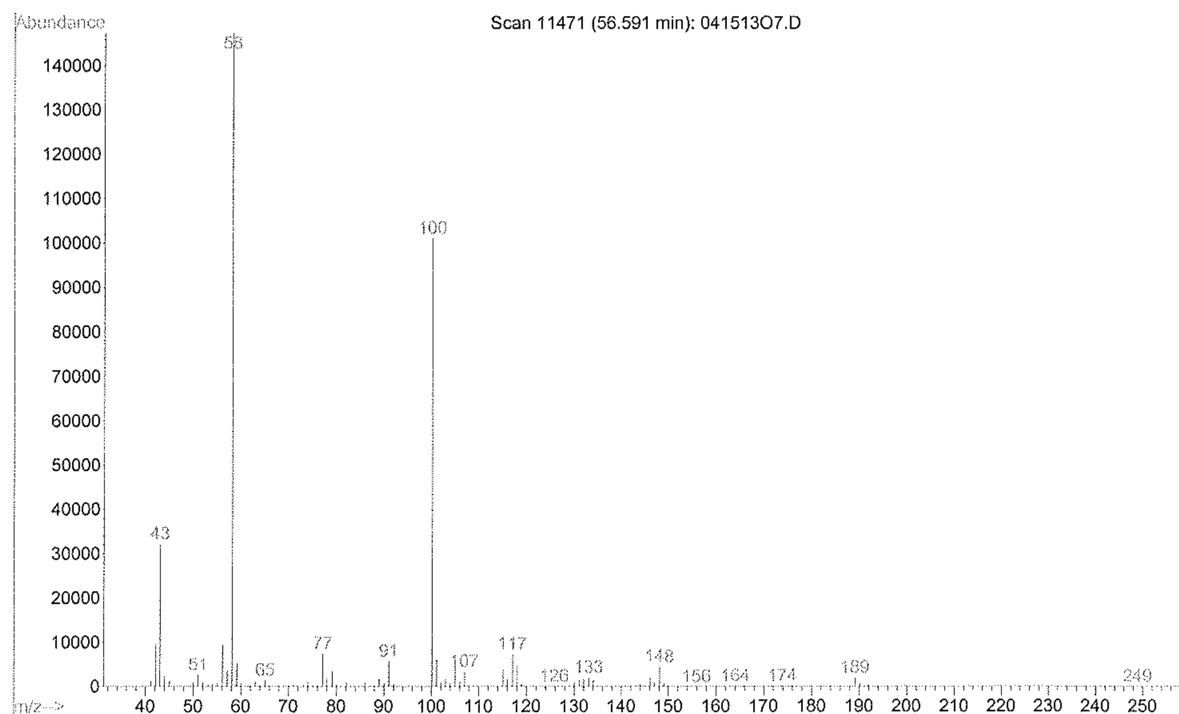


Figure 11C. Mass spectra of the peak with a retention time of 56.64 minutes. The mass spectrometry fragmenting is similar for the peaks at 55.79 and 56.64 minutes with major fragments with a mass-to-charge ratio of 43, 58, and 100.

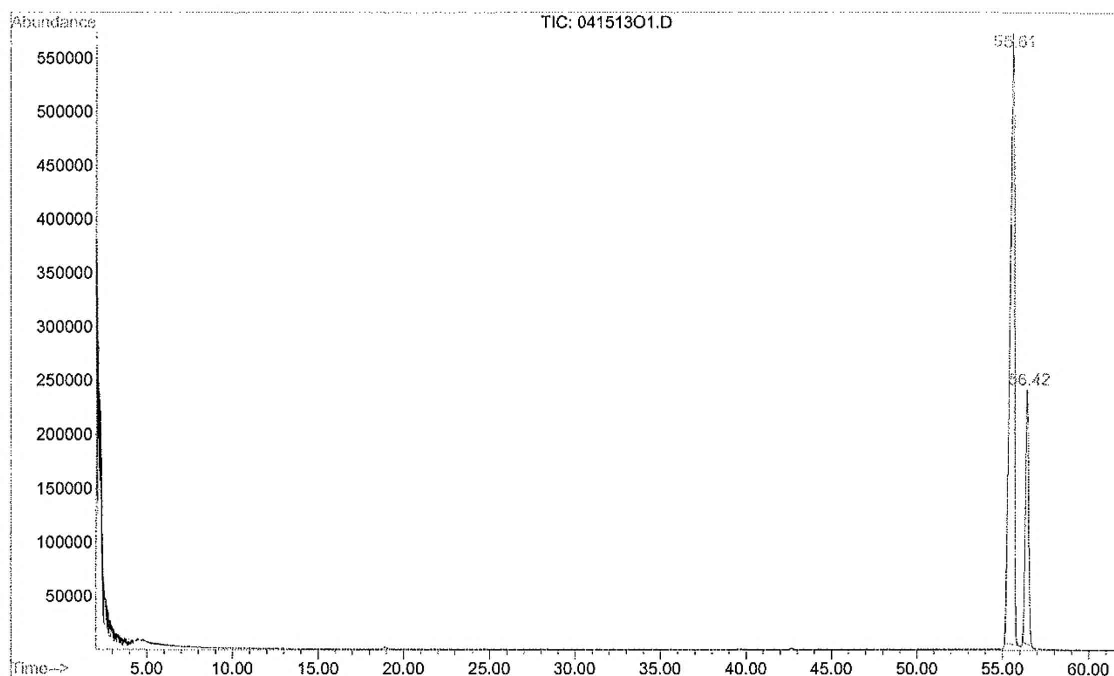


Figure 12A. Chromatograph of acetylated *(-)*-ephedrine.

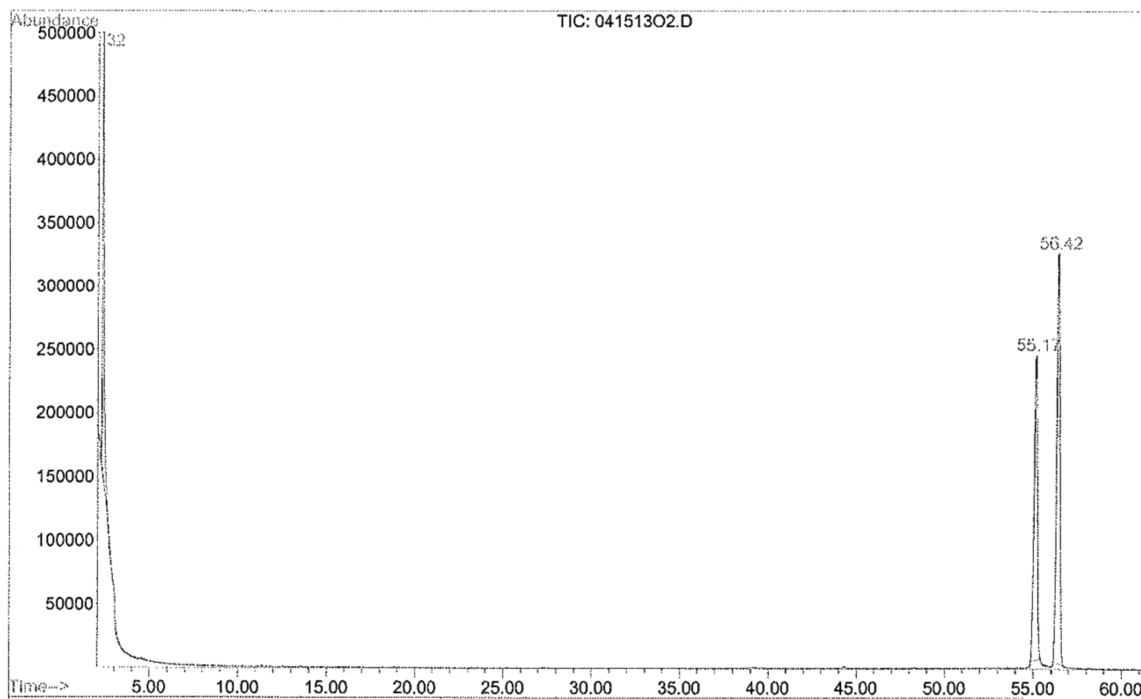


Figure 12B. Chromatograph of acetylated *(+)*-ephedrine. Both chromatographs have two major peaks at around 55 and 56 minutes when using LVCHRL10.

DISCUSSION

The goal of this research project was to develop a GC-MS Method that separated drug amine enantiomers. Different GC-MS chiral methods were developed to achieve this separation using a β-cyclodextrin column in order to minimize derivatization procedures for chiral analysis. This was first attempted with a constant temperature rate of 15°C per minute from 65°C to 225°C. Although the method was successful in obtaining two major peaks, the GC library recognized the second peak but not the first peak as ephedrine, which was confirmed by the mass spectrometry of the two peaks. The fragmentation of the two mass spectra followed different patterns confirming that the two peaks were not characteristic of two enantiomers. Enantiomers have the same fragmentation in mass spectrometry because their structures are essentially the same (Paul, et. al., 2004).

To attempt better separation the LVCHIRAL, LVCHIRL2, and LVCHIRL3 methods were developed from similar work published by Drake in 2011 where a γ-cyclodextrin chiral column and achieved chiral separation. When using these methods in the analysis of the two ephedrines, there was a lack of peaks in each chromatograph, they were not eluted. When comparing the temperature programming and the chromatographs while using DAICHIRL, the retention times of the major peaks were 9.42 and 9.72 minutes. The ephedrine mixture was eluted between 185°C and 200°C indicating that the temperature parameters for the LVCHIRAL, LVCHIRL2, and LVCHIRL3 were not appropriate for the β-cyclodextrin column. This is due to the difference in chiral columns; β- and γ-cyclodextrin differ in their number of D-glucose molecules and as a result, temperature parameters that are efficient for one column are not for the other column.

LVCHIRL4 was adapted from the work published by Wang on the chiral separation achieved using a β-cyclodextrin column in 2006. The use of this method resulted in two major unresolved peaks at 44.91 and 45.63 minutes with slight tailing effects that are due to unwanted interactions between the β-cyclodextrin and the ephedrine. When this method was used to analyze the individual enantiomers, the enantiomers were eluted at slightly different times but had broad, unresolved peaks with tailing. Following this analysis, it was hypothesized that hydrochloride led to unwanted interactions. The free-base extraction of the two ephedrines was performed to eliminate these issues.

When the LVCHIRL4 method was used to analyze chiral separation of the free base ephedrine mixture, there was no separation leading to the development of LVCHIRL5, LVCHIRL6, and LVCHIRL7. These methods were designed with similar temperature programming as LVCHIRL4 with alternating temperature rates. When comparing the chiral separation using these three methods LVCHIRL7 led to resolution, resulting in two unresolved peaks at 19.23 and 19.99 minutes. The LVCHIRL9 method was adapted from method parameters included in the Restek chiral chromatography guide for the separation of menthol enantiomers. Although this method was successful for menthol, ephedrine and menthol lack structural similarities and did not lead to chiral ephedrine separation.

Derivatization with acetic anhydride of the two ephedrines was performed to minimize hydrogen bonding between the hydroxyl and amine groups on the ephedrine enantiomers and hydroxyl groups on the β-cyclodextrin and lead to more efficient separation. To analyze the acetylated ephedrine, LVCHRL10 was adapted from the LVCHIRL4 method using the same temperature rate. The starting and end temperature were increased to ensure detection of any peaks retained for longer amount of time. When the mixture of the two acetylated ephedrine

enantiomers were analyzed using LVCHRL10, there were two major peaks at 55.79 and 56.64 minutes. Initially, it was believed that the two peaks belonged to two acetylated enantiomers. However, when each acetylated ephedrine enantiomer was analyzed using LVCHRL10, the same two peaks were present in each acetylated enantiomer. Based on these results and the similarities in the fragmentation of the two chromatograph peaks mass spectra, the two peaks are two forms of ephedrine and are either conformational or constitutive isomers. It is more likely that the two peaks belong to two constitutional or structural isomers that were produced by the acetylation of the hydroxyl and amine groups during the derivatization procedures, as seen in Figure 13.

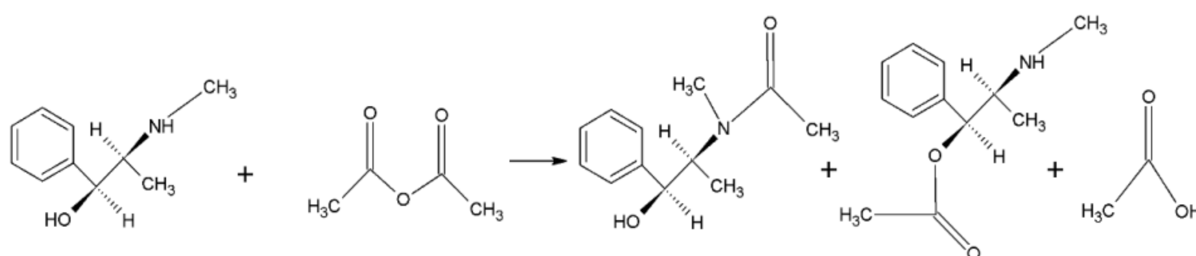


Figure 13. Mechanism of Acetylation of *(1R,2S)*-Ephedrine. The hydroxyl and amine groups of ephedrine were acetylated through two different synthetic routes through the use of acetic anhydride. These are the structure of the two derived structural isomers.

Based on the results and analyses it cannot be determined whether chiral separation can or cannot be achieve solely through the use of a chiral β-cyclodextrin column. The results have indicated that derivatization procedures are highly effective and cannot be minimized before the use of β-cyclodextrin for enantiomeric separation, at least for ephedrine. These results do indicate that the derivatization and temperature programming are heavily involved in the resolution of the chromatography peaks. Further chiral analysis may be continued with the use of a bulky acetylation reagent that is reactive solely with the amine functional group of ephedrine. Derivatization with pentafluoropropanoic anhydride (PFPA) or heptafluorobutyric anhydride (HFAA) would be more effective in the acetylation of the amine group and not the hydroxyl group because the larger structure of these two acetylation reagents lead to steric hindrance.

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