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Analysis of cycloserine and related compounds using aqueous normal phase chromatography/mass spectrometry

Joseph J. Pesek*, Maria T. Matyska, Andy Dang

Department of Chemistry, San Jose State University, San Jose, CA 95192, USA

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ABSTRACT

A new approach is evaluated for the analysis of cycloserine, a strongly hydrophilic drug. The method utilized is aqueous normal phase chromatography with a silica hydride-based stationary phase and mass spectrometry for detection. The samples are analyzed to determine the number of components and they are identified when possible. In addition, the composition change is monitored with respect to time and sample solvent. Analyses using both gradient and isocratic conditions are presented. The repeatability of inter- and intraday analyses is also determined.

1. Introduction

Cycloserine is a highly polar general antibiotic which can also be used in the treatment of pulmonary tuberculosis [1]. Until recently it has not been in wide-spread use for the treatment of TB due to its toxicity. With more drug-resistant strains of tuberculosis emerging, cycloserine treatment is becoming more common [2]. Bioequivalency has been demonstrated when cycloserine is administered in different formulations [3]. It also exhibits the ability to increase the levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) [4–6] as well as inhibit the pyridoxal-5′-phosphate (PLP) enzyme GABA-aminotransferase [4,7,8]. The interaction of cycloserine with a number of other PLP enzymes has also been studied [9,10].

A number of analytical approaches have been developed for the detection and quantitation of cycloserine, with most of the more recent methods based on HPLC. To date all HPLC methods have been based on using a reversed-phase approach. This necessitates the use of an ion-pairing reagent [11–13] or derivatization [14–16] in order to retain the very hydrophilic compound cycloserine and its analogs on a column such as C18. These methods have limitations and are not the optimal approach to the analysis of polar compounds. Derivatization adds an extra step to the determination of the target compounds and can lead to longer analysis times. In addition, some derivatization processes are not quantitative resulting in variable conversion efficiencies that can lead to substantial errors. Ion-paring is often accomplished by the use of mobile phase components that are not compatible with detection by mass spectrometry. MS is more frequently becoming the technique of choice for detection because it can provide more reliable compound identification, especially in complex matrices.

A more compatible approach for the analysis of hydrophilic compounds such as cycloserine is aqueous normal phase (ANP) chromatography using silica hydride-based stationary phases [17,18]. ANP is a normal phase method similar to hydrophilic interaction liquid chromatography (HILIC) where retention of polar compounds increases as the organic component of typical binary mobile phases with water increases. Thus if the mobile phase consists of acetonitrile/water, retention increases as the amount of ACN increases. However, ANP using silica hydride-based stationary phases does not suffer from the drawbacks associated with many HILIC analyses such as poor reproducibility, long equilibrium times between gradient runs and poor column lifetime. In addition, all silica hydride materials can retain hydrophobic compounds when used in the reversed-phase mode [19–21], a feature not found to any appreciable extent on HILIC phases. A wide variety of applications have already been reported for polar compounds on silica hydride phases including amino acids, organic acids, carbohydrates [18], nucleotides [22,23] and peptides [24]. A number of investigations have also included biological and physiological matrices [25–27]. This investigation explores the potential use of ANP for monitoring drugs and their metabolites by focusing on cycloserine that has to date only been analyzed chromatographically with reversed-phase methods.

* Corresponding author. Tel.: +1 408 924 4950; fax: +1 408 924 4945.
E-mail address: joseph.pesek@sjsu.edu (J.J. Pesek).

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2. Experimental

2.1. Materials

The silica hydride stationary phase used in this study was the Cogent Diamond Hydride (DH) material ($d_P \sim 4.0\,\mu m$) in 150 mm × 2.1 mm columns (MicroSolv Technology, Eatontown, NJ, USA). The phase contains a small amount of an organic moiety (~2% carbon as reported by the manufacturer) on a silica hydride surface. The analytes and mobile phase additives used in this study were purchased from Sigma–Aldrich (Milwaukee, WI, USA) in the highest purity available. Mobile phase solvents used were HPLC grade.

2.2. Instrumentation

The HPLC was an Agilent (Little Falls, DE, USA) 1200SL Series LC system, including degasser, binary pump, temperature-controlled autosampler and temperature-controlled column compartment. The mass spectrometer system was an Agilent (Santa Clara, CA, USA) Model 6220 MSD TOF with a dual sprayer electrospray source (ESI).

2.3. Methods

Stock solutions of the analytes were made in deionized (DI) water in the range of 0.2–0.7 mg/mL. Sample solutions were made by diluting the stock 1:100 in 50:50 acetonitrile/water or 2:1 methanol/water containing the mobile phase additive (acetic acid or formic acid) used in the analysis. The flow rate was 0.4 mL/min. The column temperature was 20 °C.

3. Results and discussion

3.1. Aqueous normal phase retention

The column used in this investigation referred to as the Diamond Hydride (DH) has been successfully used for the retention of a broad range of polar compounds from hydrophilic metabolites [18] to peptides [24]. The unique surface structure of this material consists of predominantly (~95%) Si–H moieties [17,28] with a small amount of a bonded alkyl group. For any polar compound that might potentially be retained on the DH column, it is first necessary to establish ANP behavior. The results of such a test are shown in Fig. 1. In Fig. 1A significant retention can be observed for an underivatized cycloserine standard under isocratic conditions using a mobile phase consisting of 50:50 (v/v) DI water/2-propanol with 0.05% acetic acid and 97:3 acetonitrile/DI water with 0.1% acetic acid. The additive, acetic acid, is MS compatible. Detection is in the positive ion mode using the $(M+H)^+$ ion at m/z 103.0502. Fig. 1B establishes the presence of the ANP mode since retention is decreased as the amount of water (the most polar component) in the mobile phase is increased. An interesting point here is that retention in the normal phase mode is still substantial with more than 60% water in the mobile phase. This is in contrast to most HILIC methods that usually require significantly more organic component in the mobile phase to obtain retention of polar compounds [29–31]. Acetic or formic acid are present in the mobile phase as a source of protons for electrospray ionization to produce the $[M+H]^+$ ion for MS detection. Isopropanol in the A solvent is used to keep the DH column relatively free of contaminants, which is particularly useful when analyzing biological samples.

While peak shape and efficiency are acceptable in the isocratic results shown in Fig. 1A and B, substantial improvement can be obtained by using a gradient elution as shown in Fig. 1C. The efficiency increases from 50,000 to 80,000 plates/m (A_S 0.8–1.5) with isocratic elution to approximately 500,000 plates/m with an asymmetry factor (A_S) of 1.0 under gradient conditions. Since silica hydride phases equilibrate rapidly when returning to the starting conditions (<5 min) there is no substantial increase in analysis time especially considering that by adjusting the gradient profile elution of the analyte can be achieved in a shorter time. Decreasing the chromatographic window is not a problem even for more complex samples when using MS for detection in the extracted ion chromatogram (EIC) mode since this greatly reduces the need to separate all components. This MS benefit is further improved

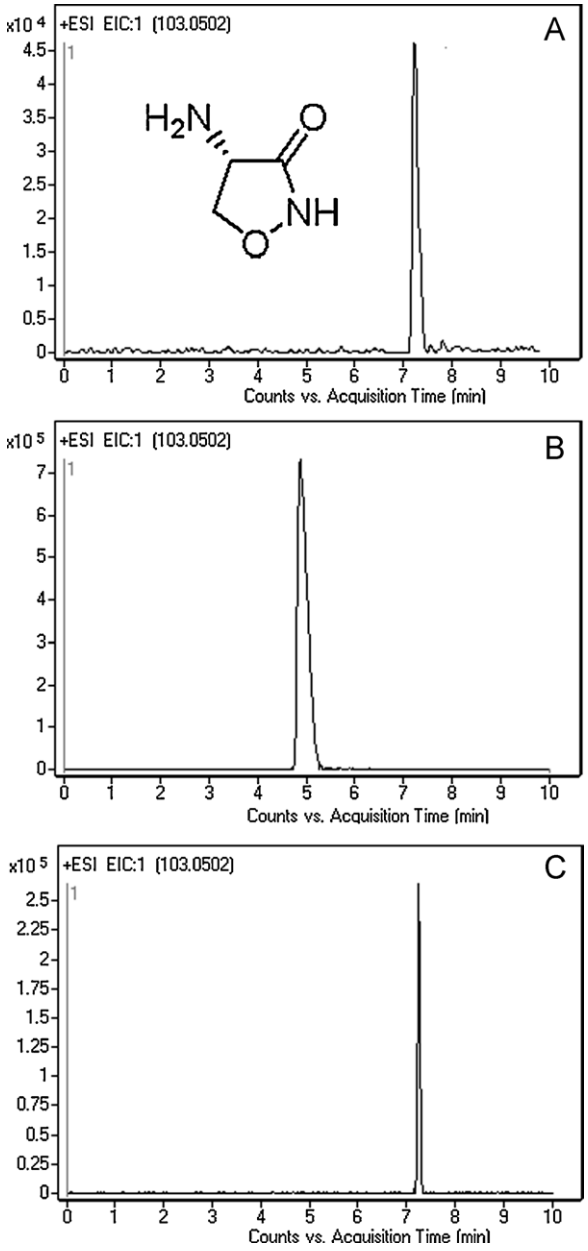

Fig. 1. Retention of cycloserine standard on the Diamond Hydride column. (A) Isocratic at 50:50 A/B; (B) Isocratic at 60:40 A/B; and (C) gradient under the following conditions: 0.0 min at 70% B, 0.0–5.0 min to 30% B, 5.0–6.0 min at 30% B, 6.0–7.0 min to 70% B. Mobile phase: A, 50:50 DI water/2-propanol with 0.05% acetic acid and B, 97:3 acetonitrile/DI water with 0.1% acetic acid. Detection by MS at m/z 103.0502.

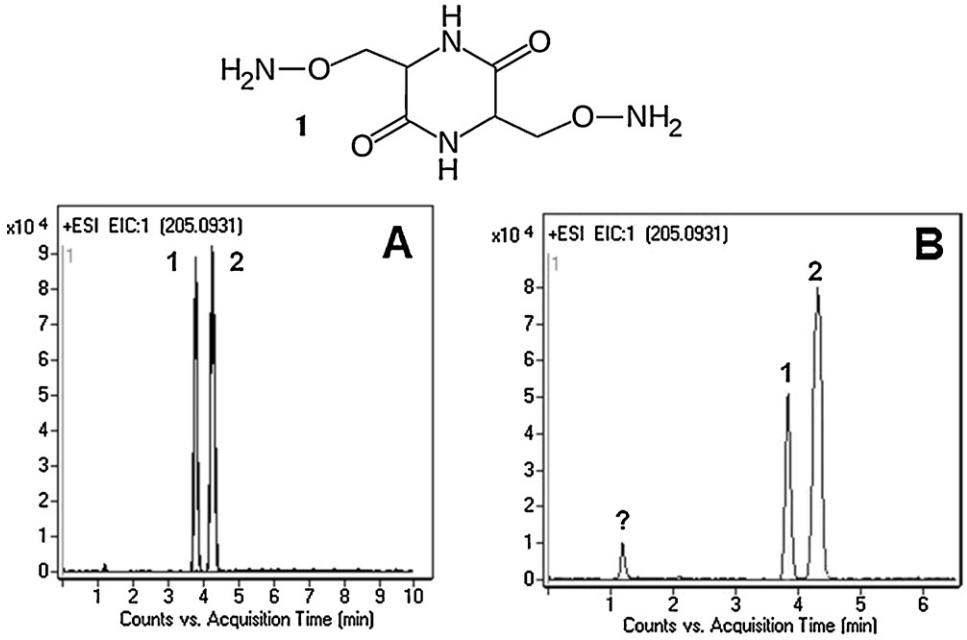

Fig. 2. Extracted ion chromatograms of cycloserine (**2** @ *m*/*z* 103.0502) and cycloserine dimer (**1** @ *m*/*z* 205.0931). (A) mixture of cycloserine and dimer on day 1 and (B) mixture after 10 days. Mobile phase: A, 50:50 DI water/2-propanol with 0.1% formic acid and B, 97:3 acetonitrile/DI water with 0.1% formic acid. Gradient: 0.0 min at 70% B, 0.0–2.0 min to 20% B, 2.0–6.0 min at 20% B, 6.0–7.0 min to 70% B.

when a more accurate mass analyzer such as a time of flight (TOF) instrument similar to the one in this investigation is used.

4. Cycloserine dimer

As reported previously [12,13] cycloserine dimer is the main impurity/degradation compound found in seromycin (cycloserine) drug products. Fig. 2A shows five overlaid chromatograms for the separation of these two compounds with MS detection at *m*/*z* 205.0931 for the dimer using a gradient that is steeper and goes to a lower percentage of acetonitrile (ACN) than the one used in Fig. 1C. This result demonstrates that the two primary analytes can be separated in less than 5 min, faster than reported with reversed-phase, polar endcapped or polar embedded columns, with peaks having both high efficiency and symmetry. In contrast to one of the earlier studies [12] comparing different columns for this separation, there is no problem with rapidly achieving reproducible results since there are no spurious column effects that require long equilibrium times or tedious conditioning steps. Thus aqueous normal phase chromatography provides superior performance to any other separation method reported to date for the analysis of cycloserine and its dimer.

The long-term stability of these two compounds was evaluated by testing the solution in Fig. 2A after 10 days. The result is shown in Fig. 2B. In relation to peak intensities in Fig. 2A, cycloserine has decreased about 10% while the dimer has lost more than 40% of its signal indicating that the dimer is degrading more rapidly than the primary analyte.

4.1. Other impurities and degradation products

Previous studies [12,13] have determined that in addition to cycloserine and the cycloserine dimer, other compounds, either degradation products or impurities in the original formula, were also present in the samples analyzed. However, because the mode of detection was UV spectroscopy, no information about these compounds was provided. Using the ANP method on the DH column and MS detection, three additional peaks were found in the total ion chromatogram (TIC). The *m*/*z* ratios were determined by examining the mass spectra extracted from each of these peaks. The *m*/*z* values were determined to be 106, 245 sand 285. Fig. 3A shows the overlaid EICs for the two high molecular weight (MW) weight compounds as well as those of cycloserine and the dimer. While the dimer is only slightly less polar than cycloserine, the two higher MW compounds elute considerably earlier indicating that while they are polar since ANP retention is observed, they are less hydrophilic than the two primary analytes.

While MS provides more information than the UV detection used in previous cycloserine chromatographic studies, it does not lead to a positive identification of these high molecular weight components in the mixture. For example, for a compound having an $(M+H)^+$ ion at 245 there are a number of empirical formulas that can lead to a MW of 244. Among the possibilities are $C_8H_{12}N_4O_5$ and $C_9H_{16}N_4O_4$. Searching the Chem Abstracts molecular formula data base shows that 165 compounds are possible for $C_8H_{12}N_4O_5$ and 149 compounds are listed for $C_9H_{16}N_4O_4$. Of the total of 314 compounds at MW 244, only three have serine as a component in their structure: serine, N-[[(3-methyl-1,2,4-oxadiazol-5-yl)methyl]amino]carbonyl; serine, alanyl-, diazoacetate; and serine, N-[(2R)-4-azido-2-methyl-1-oxobutyl]-,methyl ester. A similar search for MW 284 results in two reasonable possibilities: $C_{10}H_{14}N_5O_5$ with 15 compounds and $C_{11}H_{16}N_4O_5$ consisting of 280 compounds. However, none of the 15 compounds from $C_{10}H_{14}N_5O_5$ have serine as part of the structure but three compounds from $C_{11}H_{16}N_4O_5$ can be considered as candidates for the $(M+H)^+$ ion: serine, N-(N-acetyl-L-histidyl); homoserine, N-[3-(4-amino-2-oxo-1(2H)-pyrimidinyl)-1-oxopropyl]; and serine, N-histidyl-, acetate. These two compounds are not impurities but come from degradation of the original components in the mixture cycloserine and cycloserine dimer. This is evident in Fig. 3B which shows these compounds have increased in concentration after ten days. While changes from the original compounds do not necessarily mean the original serine structure is preserved, it is certainly a likely possibility.

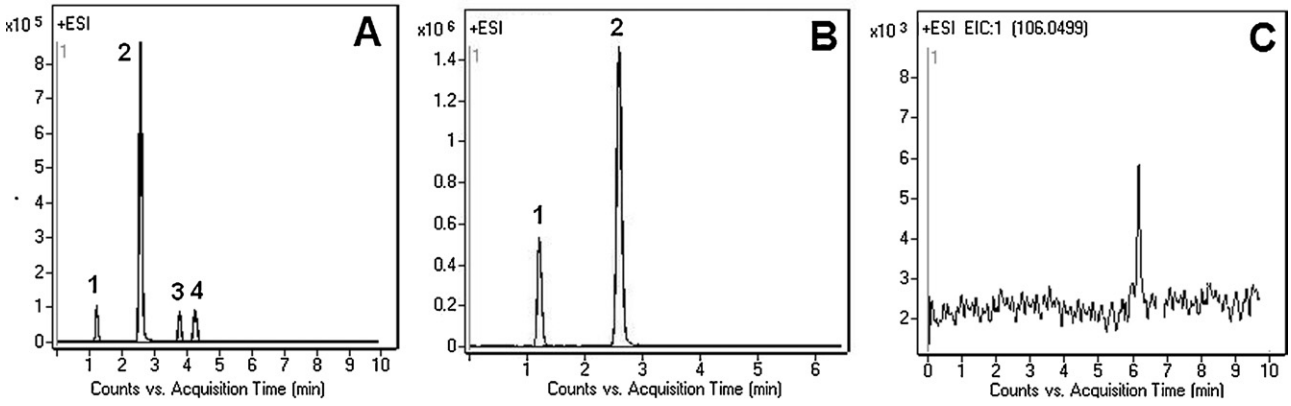

Fig. 3. Extracted ion chromatograms of impurities/degradtion products in cycloserine samples. (A) One day after preparation. (B) Two high molecular weight degradation products after ten days. (C) EIC of Serine one month after sample preparation. Mobile phase and gradient same as Fig. 2. Peaks: **1** unknown at *m*/*z* 285; **2** unknown at *m*/*z* 245; **3** cycloserine dimer at *m*/*z* 205; and **4** cycloserine at *m*/*z* 103.

One of the earlier studies [13] also reported the possibility that a small of amount of serine is present in cycloserine samples. With MS detection the presence of a particular compound in a sample is much easier to verify. Fig. 3C shows the EIC for *m*/*z* 106.0–106.2 corresponding to the $(M+H)^+$ ion for serine. A measurable peak is observed for serine at a retention time of about 6 min having an intensity that is about 10% of cycloserine and its dimer and about 1% of the degradation product with *m*/*z* 244. In this study serine was not detected in measurable quantities in the sample until more than one month after it was prepared. Thus this result absolutely confirms the presence of serine as well as its relatively low abundance in comparison to the other compounds found in cycloserine samples.

4.2. Sample solvent effects

Another factor in cycloserine analysis is the choice of sample solvent. Fig. 4 demonstrates that the major products formed vary depending on the sample solvent. The composite EICs for the four primary components are overlaid in the figure. In Fig. 4A the sample is dissolved in 2:1 methanol/DI water. In this solvent there is no dimer present and the most abundant compound is cycloserine. In contrast, when the sample is placed in 100% acetonitrile containing 0.1% formic acid, there is only a small amount of cycloserine and dimer present with the unknown impurity at *m*/*z* 245 being the predominant species.

4.3. Repeatability and quantitation

One of the previously reported [17,18] advantages of ANP using silica hydride stationary phases for the retention of hydrophilic compounds is the high repeatability of consecutive chromatographic runs. Fig. 2A is a test of day-to-day reproducibility. Two runs were made on the first day, then the column was used for additional determinations on cycloserine samples, and finally three injections of the same sample were done on the second day. The overlay of the five chromatograms shows a high degree of reproducibility for retention times with an RSD of 0.3%. This is a gradient run and the equilibration time between consecutive runs is five minutes. Fig. 3B is the overlay of five consecutive runs using a gradient method and a 5 min column equilibration before the next analysis. A high degree of repeatability is achieved for this test, similar to the example in Fig. 2A (RSD ~ 0.3%). These results further demonstrate the ruggedness of ANP methods on silica hydride columns.

Although the objective of this study does not involve the development of a validated method, some indication of the quantitative

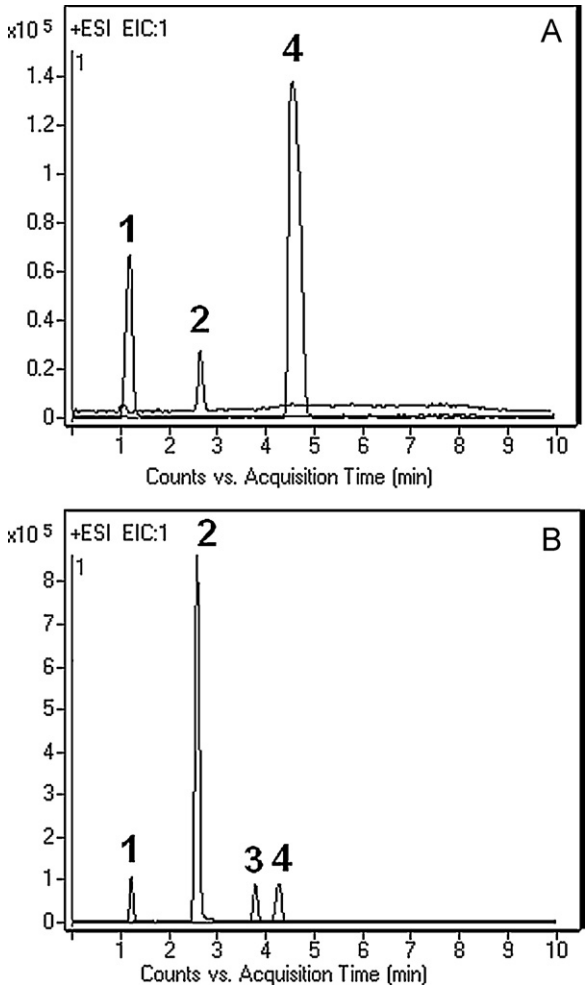

Fig. 4. Variation of sample components in cycloserine samples after one day with different sample solvents. (A) Sample in MeOH/water 2:1 and *B) Sample in 100% acetonitrile + 0.1% formic acid. Gradient and mobile phase same as Fig. 2. Peaks same as Fig. 3.

capabilities of the aqueous normal phase approach would be beneficial for potential users. A linear relationship following the equation $2E + 6x - 280{,}000$ was obtained for the determination of cycloserine over the concentration range of 0.2–1.0 μg/mL having an R^2 value of 0.993. The limit of detection is estimated to be 0.1 μg/mL. The data obtained in this study can be compared to

previously reported UV method having a detection limit of 25 μg/mL [13]. However, any method depends on the sensitivity of the detector and sample preparation procedures. Thus the LOD reported here could easily be improved by utilizing MS/MS for example.

5. Conclusions

Aqueous normal phase chromatography using a silica hydride stationary phase and mass spectrometry detection is a reliable and fast method for the analysis of hydrophilic drugs such as cycloserine. Each of the impurities or degradation products found in cycloserine are sufficiently polar to have retention in the ANP mode. While two major impurities were not directly identified, several possibilities were suggested based on their m/z values in the mass spectrum and their relationship to the serine structure. One component, serine, was also identified but in very low abundance and only after the samples were several weeks old. The repeatability of the method, both inter- and intraday, is excellent. Additional pharmaceutical, food, physiological and peptide LC/MS and LC/MS/MS applications of ANP with silica hydride-based stationary phases are ongoing.

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