Automated Purification and Analytical Reinjection of a Small Molecule Drug, Probenecid, on a Gilson LC/MS Dual Function System

Application Note PHA0413

Keywords

High Pressure Liquid Chromatography (HPLC), Mass Spectrometry (MS), preparative HPLC/MS, analytical HPLC/MS, fraction collection, fraction reinjection, probenecid, TRILUTION® LC, conditional logic fraction collection

Introduction

Combined UV and mass-based detection and fraction collection following preparative liquid chromatography (LC/MS) represents a well-established method for purifying target compounds. Typically these systems utilize both UV and MS detectors, employing conditional logic "on the fly" to determine whether collection criteria set by the user is met based on UV signals and MS selected ion signals. This technique offers the benefits of enhanced selectivity (exclusion of unwanted fractions and attendant post-collection processing) and real time as well as post-collection mass spectral confirmation data, thus overcoming the inherent limitations associated with purifying target compounds with HPLC using conventional UV detection alone.

To provide additional confirmation data on the collected fractions of interest, both preparative- and analytical-scale LC/MS can be conducted. The two methods (preparative collection and analytical) are performed on partially separate liquid channels configured using a common liquid handling platform with dual injection modules to enable full automation of the purification step and subsequent analytical reinjection step. This configuration eliminates the need for manual intervention at any point in the purification and analytical processes.

This application note describes the functionality of a Gilson LC/MS system for both the purification and identification of probenecid, a well-known uricosuric drug commonly used to treat gout.[1] The effects of the make-up solvent on the generation and optimization of mass spectra for this compound are also evaluated.

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LC/MS System Setup and Conditions

Sample Preparation

The test sample was prepared using 5 mg/mL each of ephedrine, 4-acetaminophenol, chlorpheniramine, verapamil, and probenecid dissolved in a 60:30:10 solution of water, methanol, and dimethyl sulfoxide (DMSO).

Gilson LC/MS System Components

- GX-281 Liquid Handling Platform with Platinum Z Injection Module (5 mL loop)
- Analytical Direct Injection Module
- 155 UV/VIS Detector; .01 sensitivity; 0.2 mm flow cell
- MRA Splitter set to 1:1000 split ratio (19)
- FLEXAR SQ 300 Single Quadrupole MS Detector with four SIM (selective ion monitoring) channels
- 307 Make-Up Pump

Components of the dual function system include a preparative-scale column and injector for purification and an analytical-scale column and injector for sample verification. Using TRILUTION LC 2.1 SP5 software, conditional logic fraction collection was employed to collect probenecid, while a selective reinjection function allowed for automated reinjection of the collected fraction for verification on the analytical system.

Preparative LC/MS Conditions

HPLC	MS

• Column: Phenomenex Luna 21x50 mm
• Mobile Phase:
  ▪ A: 0.1% formic acid in water
  ▪ B: 0.1% formic acid in methanol
• Gradient: 10-90% B in 7 min
• Flow rate: 20 mL/min
	• ESI mode: Positive
	• Make-up solvent: 0.1% formic acid in MeOH (0.3 mL/min flow rate)
	• Scan range: 100-500 amu
	• Scan rate: 5 scans/sec

Probenecid Fraction Collection and Reinjection onto Analytical System

The fraction containing probenecid was collected using a conditional logic method in TRILUTION® LC. Conditional logic (AND) was applied to two channels: 1) primary – UV (254 nm) slope criteria; 2) secondary – target mass criteria, i.e., probenecid SIM (m/z 285.1; level 1,500,000).

Analytical LC/MS Conditions

HPLC	MS

• Column: Phenomenex C18 6x50 mm
• Mobile Phase:
  ▪ A: 0.1% formic acid in water
  ▪ B: 0.1% formic acid in methanol
• Gradient: 10-90% B in 2 min
• Flow rate: 2 mL/min
	• ESI mode: Positive
	• Make-up solvent: 0.1% formic acid in MeOH (0.3 mL/min flow rate)
	• Scan range: 100-500 amu
	• Scan rate: 5 scans/sec

Results

The test sample was first processed on the preparative LC/MS system, with the probenecid fraction (RT: 7.512 min and m/z 285.1) collected using conditional logic on two channels (UV wavelength and target mass) (Figures 1 and 2). As indicated in Figure 2, the protonated probenecid ion ($[M+H]^+$=286.30) was assigned an abundance of 81 relative to the base peak at m/z 340.30. The base peak was suspected to be the result of a methanol cluster on the sodium adduct (m/z 308.30).

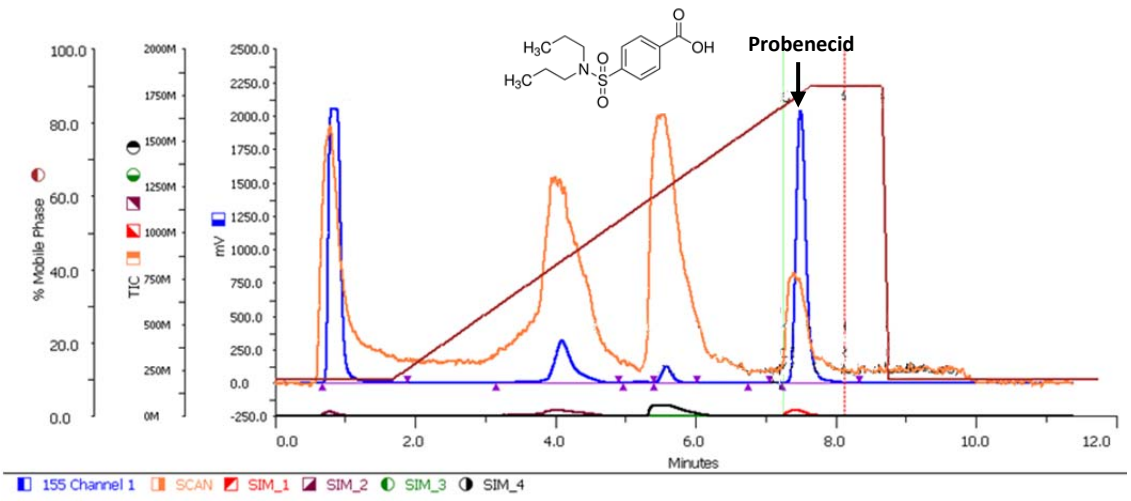
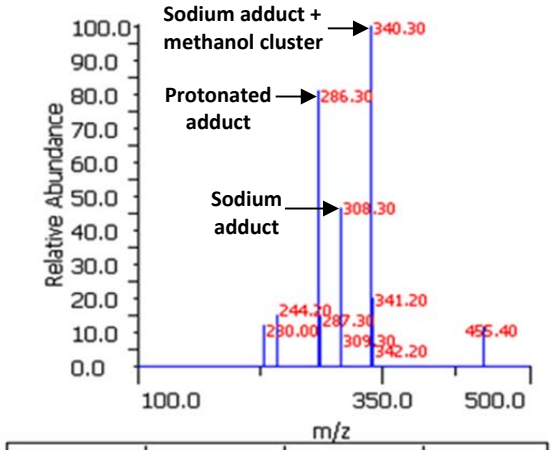


Figure 1. Chromatogram showing UV-based fractionation and detection of compounds in the test sample. Gradient: 10-90% organic mobile phase (0.1% HCOOH in MeOH) in 7 min.



340.30=100	341.20=20	230.00=12	342.20=8
286.30=81	244.20=15	455.40=12	
308.30=47	287.30=15	309.30=8	

Figure 2. Probenecid fraction collection spectrum using a 0.1% formic acid in methanol make-up solvent with a flow rate of 0.3 mL/min. The relative abundance of the protonated probenecid ion ($[M+H]^+$=286.30) is indicated in the red box.

Under TRILUTION® LC control, the collected fraction was then automatically reinjected from the fraction tube onto the analytical system for further compound verification (Figures 3 and 4). Similar to the mass spectrum shown in Figure 2, the base peak was the suspected methanol cluster on the sodium adduct at m/z 340.20. The relative abundance of the probenecid protonated adduct (48) was notably low compared to that of the identical adduct in the spectrum given by the original collected fraction.

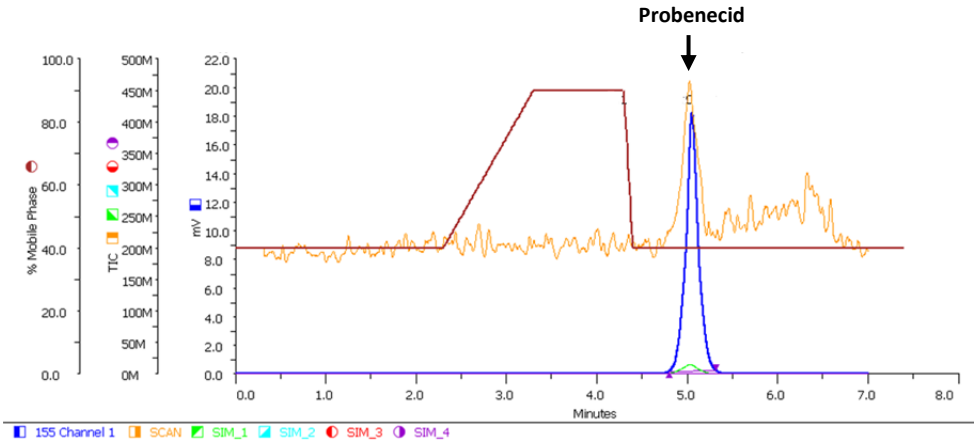
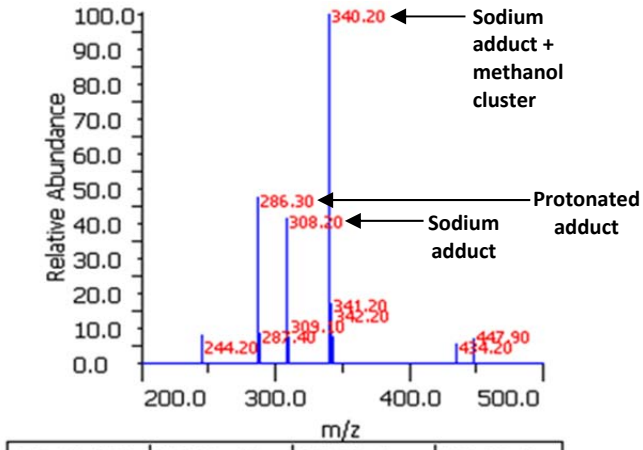


Figure 3. Probenecid fraction reinjection on an analytical system. Gradient: 10-90% organic mobile phase (0.1% HCOOH in MeOH) in 2 min.



340.20=100	341.20=17	309.10=8	434.20=6
286.30=48	287.40=9	342.20=8	
308.20=41	244.20=8	447.90=7	

Figure 4. Probenecid fraction reinjection spectrum using the make-up solvent and flow rate specified above (Figure 2). Red box indicates the protonated probenecid adduct.

As indicated above, the base peaks in the probenecid fraction collection and reinjection spectra (Figures 2 and 4) were assumed to have originated from a methanol cluster on the sodium adduct ion when the make-up solvent consisted of formic acid in methanol. To eliminate this adduct from the spectra, the experiment was repeated using a make-up solvent consisting of formic acid in water. Use of the alternative solvent yielded a nearly identical chromatogram (Figure 5), yet the mass spectra showed considerable differences between the two make-up solvents with regard to compound ionization. The peak corresponding to the methanol cluster on the sodium adduct (m/z 340.20) was absent from the spectra produced when an aqueous make-up solvent was used (Figure 6). As such, the protonated adduct was designated as the base peak with a relative abundance of 100.

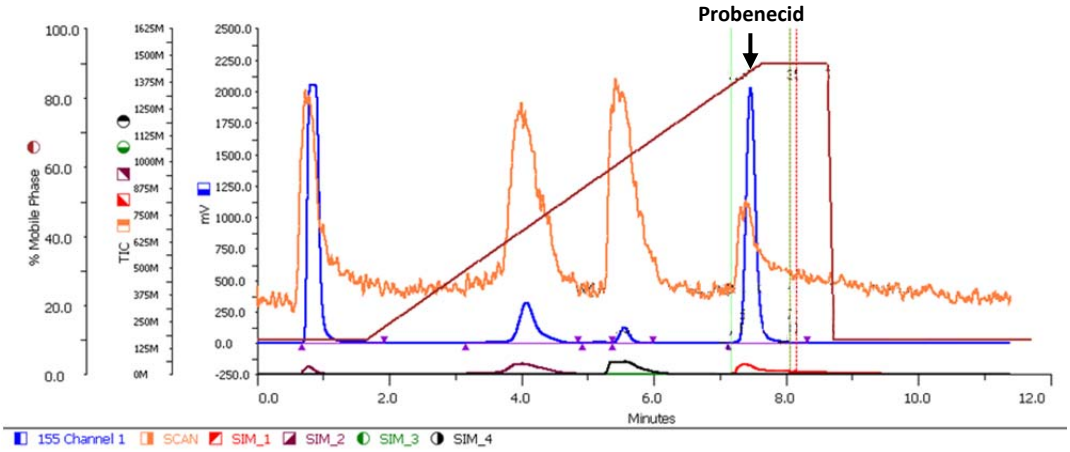
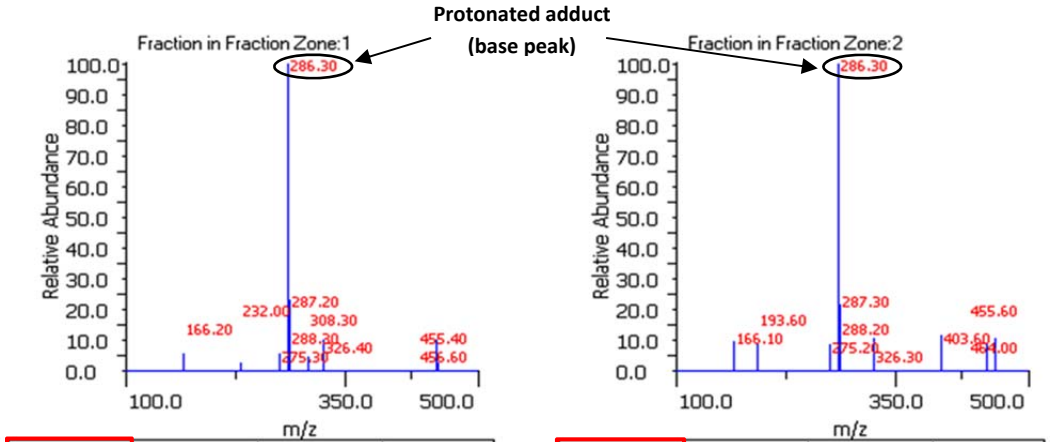


Figure 5. Fraction collection when a 0.1% formic acid in water make-up solvent was used with a flow rate of 0.3 mL/min. Gradient: 10-90% organic mobile phase (0.1% HCOOH in MeOH) in 7 min.



286.30=100	326.40=10	166.20=6	456.60=3
287.20=23	288.30=10	308.30=5	
455.40=10	275.30=6	232.00=3	

286.30=100	464.00=11	166.10=10	275.20=9
287.30=22	326.30=11	193.60=9	
403.60=12	288.20=10	455.60=9	

Figure 6. Probenecid fraction reinjection spectra when a 0.1% formic acid in water make-up solvent was used with a flow rate of 0.3 mL/min. The peak corresponding to the protonated probenecid adduct had the highest abundance (100; indicated in red boxes) and was therefore designated as the base peak.

Summary

Configuration of a standard LC/MS purification system with a second injection valve allows for integration of an analytical system with a preparative system, thereby reducing manual intervention during the reinjection step. Here, an important pharmaceutical compound, probenecid, was successfully purified from a sample mixture containing several other compounds. Based on both UV and mass spectra, the probenecid fraction was collected and reinjected onto an analytical system for absolute verification. Use of a second injector allowed the transition from preparative to analytical LC/MS to be entirely automated. Thus, the Gilson dual function LC/MS system with automated fraction collection and reinjection represents a systematic means of purifying and verifying fractions containing pharmaceutical and other compounds of interest in the laboratory, allowing laboratory chemists to focus on other activities and to avoid error-prone manual steps.

References

1. Silverman, W., Locovei, S., Dahl, G. Probenecid, a gout remedy, inhibits pannexin 1 channels. Am. J. Physiol. Cell Physiol. (2008) 295: C761-C767.