COGENT
Diamond Hydride™

Pharmaceuticals in Plasma Analyzed by LCMS

Acetyl-L-Carnitine
Codeine
Hydroxybupropion
Isotrentinon
Warfarin

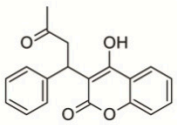
Extended Application Note

MANUFACTURED BY:
MICROSOLV TECHNOLOGY CORPORATION

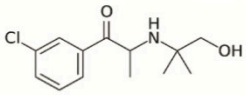
9158 Industrial Blvd NE
Leland, NC 28451

p: 1.732.380.8900
f: 1.910.769.9435

customers@mtc-usa.com
www.Cogent-HPLC.com



Warfarin



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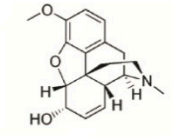

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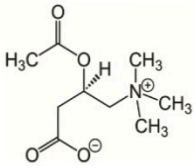
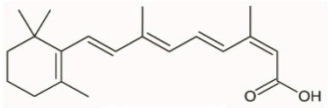


Acetyl-L-carnitine



Isotretinoin

Figure 1

Method 1:

A: DI H_2O/ 0.1% formic acid
B: Acetone/ 0.1% formic acid

Time (min.)	%B
0	90
3	30
5	30
6	90

Method 2:

A: DI H_2O/ 0.1% formic acid
B: ACN/ 0.1% formic acid

Time (min.)	%B
0	80
1	80
5	30
7	30
8	80

Method 3:

A: DI H_2O/ 0.1% formic acid
B: ACN/ 0.1% formic acid
70% A/ 30% B

Introduction

One of the most important applications of LC-MS today is the quantitative analysis of plasma samples. Pharmaceuticals and their metabolites may need to be monitored in plasma in fields such as clinical testing, forensics, and toxicology studies. Advancements in LC-MS capabilities have enabled new possibilities for these types of analyses, with lower levels of detection and enhanced MS specificity now achievable.

Despite these advancements, the analysis of blood samples is still associated with a number of obstacles. Many of the polar compounds found in these types of samples have been difficult to retain using conventional HPLC columns. Ion pair agents used to increase retention in reversed phase are unsuitable for LC-MS, and HILIC methods tend to suffer from a lack of robustness. Furthermore, the complex plasma matrix can lead to build-up of contaminants on the analytical column, which may result in increased background noise, ghost peaks, and other undesirable chromatographic phenomena.

Cogent TYPE-C Silica™ HPLC columns are able to avoid these drawbacks due to the Aqueous Normal Phase (ANP) retention mechanism. Robustness is not an issue due to the lack of a strongly associated water layer on the stationary phase surface. Build-up of matrix contaminants is less problematic as well because of the difference in retention mechanism.

The separation of various pharmaceuticals (see **Figure 1** for structures) was investigated using three types of stationary phases. The first was the Diamond Hydride™ column, which has a TYPE-C Silica™ surface with a small amount of bonded carbon. The second was the Cogent Diol 2.o™ column, which is based on TYPE-C Silica™ but has a diol bonded ligand. The third was a Cogent Bidentate C18™ column.

Experimental

Materials

Formic acid LC-MS ultra-grade, warfarin, hydroxybupropion, codeine, acetyl-L-carnitine, and isotretinoin were from Sigma–Aldrich (St. Louis, MO, USA). Deionized water (DI H2O) was prepared on a Milli-Q™ purification system from Millipore (Bedford, MA, USA). Acetonitrile (ACN) (HPLC grade) was obtained from GFS Chemicals, Inc. (Powell, OH, USA).

Instrumentation

An Agilent (Little Falls, DE, USA) 1200SL Series LC system, including degasser, binary pump, temperature-controlled autosampler, and temperature-controlled column compartment was used. The mass spectrometer system was an Agilent (Santa Clara, CA, USA) Model 6210 MSD TOF with a dual sprayer electrospray source (ESI). The first analytical column (Method 1) was a Diol 2.o™ stationary phase, which had dimensions 2.1 x 50 mm, a particle diameter of 2.2µm, and a pore size of 120Å. The second analytical column (Method 2) was a Diamond Hydride™ stationary phase, which had dimensions 2.1 x 150 mm, a particle diameter of 4µm, and a pore size of 100Å.

The third analytical column (Method 3) was a Bidentate C18™ stationary phase, which had dimensions 2.1 x 50 mm, a particle diameter of 4µm, and a pore size of 100Å. In all cases, the flow rate was 0.4mL/min and the injection volume was 1µL.

Samples Preparation

Method 1: Stock solutions of each analyte were prepared at 1mg/mL concentrations using a methanol diluent. Working solutions were then prepared from the stock solutions at concentrations of 1 microg/mL. All solutions were stored at -20 °C. Solutions used for spiking were prepared at 0.500 microg/mL concentrations. For blood samples, 0.2mL blood in a 2mL plastic tube was mixed with 0.2mL methanol and 0.2mL spiking solution. The samples were vortexed for 1 min and centrifuged for 10 min at 13,000 rpm. The final solutions were prepared by diluting 0.2mL supernatant with 0.5mL water + 0.1% formic acid.

Method 2: Plasma from healthy individuals was spiked with an acetyl-L-carnitine (ALC) standard solution and prepared for injections as described by Tallarico et al. [1]. To prepare standard curves dialysed plasma was used, to which known amounts of the analyte were added.

[1]. Carlo Tallarico, Silvia Pace, and Antonio Longo, Rapid Communications in Mass Spectrometry, Vol. 12, 403–409 (1998).

Method 3: Liquid–liquid extraction was used. A 0.5 mL aliquot of the collected plasma sample was pipetted into a 10 mL glass centrifuge tube. 4.0 mL of acetonitrile: dichloromethane (1:1, v/v) was added. The mixture was vortexed for 3 min. After centrifugation at 1330×g for 10 min at room temperature, the upper organic layer was transferred to another 10 mL centrifuge tube and evaporated to dryness under a gentle stream of nitrogen gas in a water bath at 37° C. The residues were then redissolved in 100 microL of acetonitrile and dichloromethane (1:1) mixture. 1 microL of the supernatant was injected into the LC–MS system.

Results and Discussion

Method 1: As the least polar analyte, warfarin should be first to elute. The other two compounds have amine groups, which will be ionized under the chosen mobile phase conditions. Ionized compounds generally retain better in ANP than uncharged ones. This also has the further advantage of increased sensitivity for MS detection. Warfarin has an ionizable group as well, although it may not be immediately apparent from its structure. It exhibits keto–enol tautomerization, and the keto form has an acidic hydrogen.

All three compounds were detected as the $[M+H]^+$ ions. Using the appropriate extracted ion chromatograms (EICs), a clean overlay comprising only the three analyte peaks could be obtained (**Figure 2**). Blood is a highly complex matrix yet this data is completely free of interfering peaks, as may not be the case using less sophisticated detection techniques. Specificity can be obtained by isolating each of the three EICs, but it is noteworthy that chromatographic separation was obtained as well. Baseline separation was obtained between hydroxybupropion and codeine using the Cogent Diol 2.0™ column.

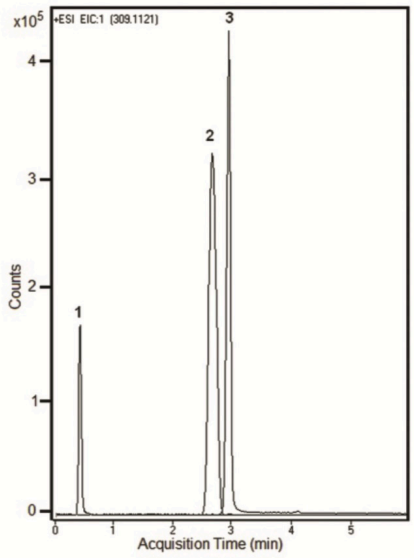


Figure 2

1. Warfarin
2. Hydroxybupropion
3. Codeine

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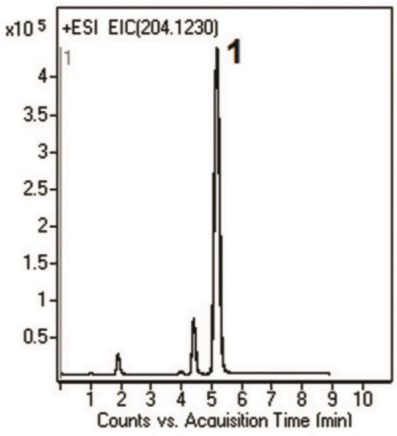

Figure 3

1. Acetyl-L-carnitine 3-run overlay

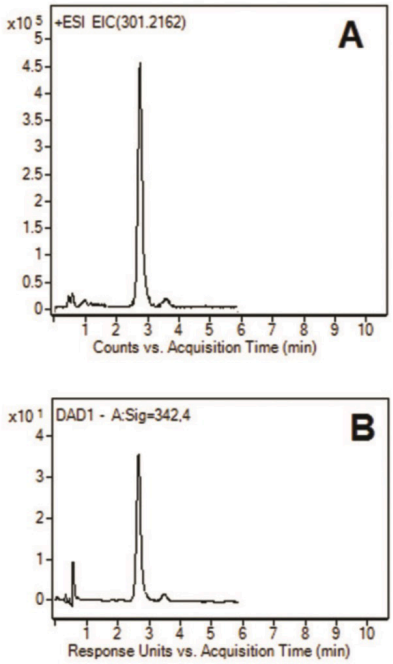

Figure 4

A. LC-MS, spiked plasma
B. UV 342 nm, standard

Method 2: Another example of a spiked plasma sample can be shown for acetyl-L-carnitine (ALC). This time, the Cogent Diamond Hydride™ column was used. As a permanently charged cationic compound, ALC can present problems to the chromatographer. Compounds like this may tail excessively on conventional silica-based columns. This is because the quaternary amine group can electrostatically bind with residual silanol groups on the stationary phase surface. Columns based on TYPE-C Silica™ on the other hand are virtually free of these silanols. Therefore, the associated tailing can be avoided. Indeed, this is what was observed in the data (**Figure 3**).

Method 3: In addition to ANP, Cogent™ columns can be used for reversed phase analyses of plasma samples as well. Isotretinoin, used to treat various skin conditions, is one example. Using the Bidentate C18TM column, separation of various peaks from the plasma matrix was obtained (Figure 4). Analysis by LC-MS (**Figure 4A**) is generally preferred for biological samples due to the complexity of the matrix, but HPLC-UV may be used for method development with standards (**Figure 4B**). Comparing the two figures, the EIC from the spiked plasma sample is so free of interferences that it looks very similar to the UV-based data using a standard.

If you are analyzing plasma samples by ANP and experience issues with build-up of contaminants in the column, try adding 50% methanol or isopropanol to the A solvent. This helps elute these kinds of compounds from the column on a run-by-run basis. You can end your gradient with 100% solvent A and hold for a few minutes to act as a washing step. If you don't want to have this solvent as part of your method, incorporate a wash step every 10 injections or so which consists of 50% DI water and 50% methanol or IPA.

Conclusion

Using Cogent TYPE-C Silica™ based columns, plasma samples can be readily analyzed either by reversed phase or ANP. Problems associated with lack of specificity can be overcome by the columns' ability to retain either polar or nonpolar compounds as well as by the detector properties themselves. Robustness issues due to build-up of plasma matrix components on the column are less of a concern with Cogent™ phases due to the low amount of surface silanols. If any such issues occur, convenient washing protocols can be used, either by incorporating them into the method itself or in separate washing steps.