Development of new stationary phase for separation of polar lipids and related analogs by Supercritical Fluid Chromatography (SFC)

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Abstract

The structural similarities between polar lipids, such as cholesterol and related analogs, have posed chromatographic and spectrometric challenges to analysts interested in quantifying these potential biomarkers. Current liquid (HPLC) and gas (GC) chromatographic methods usually require long runtimes or sample derivatization to effect adequate resolution of the analytes. Supercritical fluid chromatography (SFC) is a technique that offers both the solvation advantages of HPLC and the high selectivity of GC, but with broader compound applicability. Baseline separation of cholesterol and lathosterol was achieved using two 2-ethylpyridine columns, and improved resolution of the peaks was achieved on a single COSMOSIL piNap (naphthalene) column. However, while both columns provide unique separations, neither completely resolved the other analogs, such as 7-dehydrocholesterol and 5- α -cholestan-3-one. The development of a Quinoline column, which combines the pi-pi interactions and structural rigidity of the piNap phase and the hydrogen bonding of the pyridine phase, demonstrates superior selectivity of the analogs with a reduced overall run time. Semi-quantative results on a single-quad 1100 MSD will also be presented.

Introduction

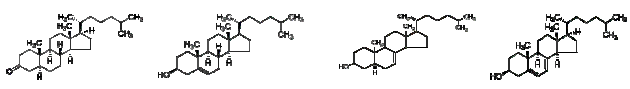
Plasma cholesterol levels have been shown to be correlated with the incidence of cardiovascular events, and are of major medical concern.[1,2] The plasma cholesterol level depends on two different processes: (i) cholesterol production by the liver (which is partly under genetic control) and (ii) cholesterol absorption by the intestines.[3–6] The hepatic production rate of cholesterol can be determined by the analysis of the plasma level of various precursors, such as lathosterol and desmosterol. Commonly used methods for cholesterol metabolite analysis are gas chromatography/mass spectrometry (GC/MS),[4,7,8] high-performance liquid chromatography/ ultraviolet (HPLC/UV),[8,9] and thin-layer chromatography (TLC).[9] The main disadvantage of GC methods is the need for sample derivatization, which makes the analysis complicated and time-consuming. HPLC/UV and TLC methods have the disadvantage of relatively poor sensitivity and selectivity towards the sterol molecules. More recently atmospheric pressure ionization mass spectrometry (API-MS) coupled to liquid chromatography (LC) has been used and this provides very good selectivity and sensitivity for the analysis of lipids from various matrices.[8,10–13] Supercritical fluid chromatography (SFC) utilizes a non-polar compressed gas at room temperature, such as carbon dioxide (CO_2), and a polar modifier, i.e. methanol, to elute analytes from the stationary phase. Due to the properties of CO_2, diffusivity of the analyte into the stationary phase is increased and the viscosity of the mobile phase is reduced, leading to a lower column pressure drop when compared to HPLC. Dues to the structural similarities of cholesterol metabolites and the need for short analysis time, SFC is ideally suited for this analysis. In order to maximize the selectivity, column chemistries with both polar and pi-pi interactions will be needed. This study demonstrates the selectivity differences of several commercial columns of this type and introduces the development of a novel phase ideally suited for sterol analysis.

Experimental

Chemicals and Reagents

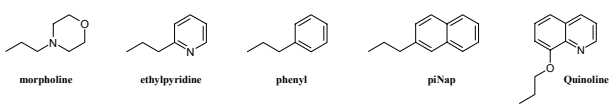
Cholesterol 99% (3β-Hydroxy-5-cholestene, CAS#57-88-5) and Lathosterol (3β-Hydroxy-5α, 7-cholestene, CAS#80-99-9), 5 α -Cholestan-3-one (Crystalline, CAS#566-88-1), 7-Dehydrocholesterol (Provitamin D_3, CAS#434-16-2), and CHROMASOLV® Plus HPLC grade methanol were purchased from Sigma-Aldrich (Saint Louis, MO). Carbon dioxide and nitrogen are bulk grade and were purchased from AirGas West (Escondido, CA). The CO_2 was purified and pressurized to 1500 psig using a custom booster and purifier system from Va-Tran Systems Inc. (Chula Vista, CA) and supplied to all SFC instruments in the lab.

Figure 1 : Cholesterol and metabolites



7-Dehydrocholesterol | Cholesterol | 5α-Cholest-7-en-3β-ol | 5α-Cholestan-3-one

Figure 2: Stationary phases used



morpholine | ethylpyridine | phenyl | piNap | Quinoline

Sample Preparation

1-mg mg of each sterol was dissolved in separate 1-mL volumetric flasks and diluted to volume with methanol. 100µL of each sample was combined in a single 1-mL volumetric flask and diluted with methanol. Serial dilutions were performed to obtain solutions containing 100-µg/mL, 50- µg/mL, 10- µg/mL, 1- µg/mL, 0.5- µg/mL of each sterol.

Instrumentation

The SFC/MS system used in this experiment is a customized supercritical fluid chromatograph, configured from instruments obtained from several manufacturers. The mass spectrometer used is a single quadrupole LC/MSD with an APCI source (Agilent, Palo-Alto, CA). A Berger analytical supercritical fluid chromatograph consisting of a dual pump fluid control module (FCM-1200), thermal control module (TCM-2000), and an Agilent 1100 DAD (Thar Instruments, Pittsburgh, PA) was used. The SFC system is also connected to a CTC HTS PAL autosampler (Leap Technologies, NC). The CTC HTS PAL was equipped with a 25 µL syringe and 20 µL fixed loop and the control method includes a routine to vent the loop of liquid CO_2 prior to sample introduction to prevent sample loss.

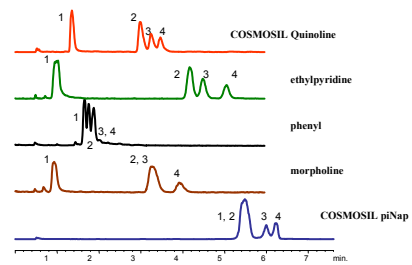
All data were acquired using Agilent 32-bit ChemStation™ (version B.03.01 [317]) in combination with Berger SFC Massware MSD™ software (version 5.4) from Thar Instruments Inc. The effluent of the SFC is split to the MSD using a tee (Valco, Houston, TX) and PEEKsil capillary tubing (Upchurch Scientific, Oak Harbor, WA); 50 cm long with an internal i.d. of 50 µm.

Analysis Conditions

Analysis was performed using COSMOSIL piNap and Quinoline columns (Nacalai USA, Inc., San Diego) and ZymorSPHER Pegasus (2-ethylpyridine), Morpholine, and Phenyl SFC columns (Zymor, Inc., Wayne, NJ) with 5 µ particle and 100Å pore size. The flow rate was 4 mL/min with the outlet backpressure set to 140 bar. The injection volume was 5 uL. The mobile phase composition was varied and the column temperature was set to 55° C. Column dimensions were 4.6mm x 150mm, unless otherwise noted. See figure captions for additional details.

Discussion

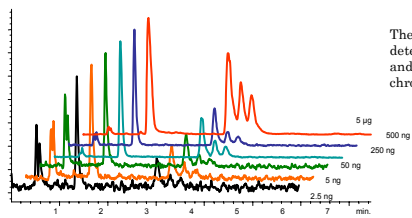
Figure 3: Chromatograms of the four sterol compounds on each column using 2% methanol as modifier.



1. 7-Dehydrocholesterol
2. Cholesterol
3. 5 α -Cholest-7-en-3 β -ol
4. 5 α -Cholestan-3-one

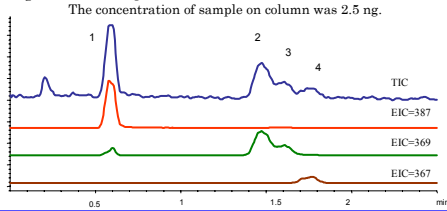
Figure 3 shows the separation of the four components on the quinoline phase was similar to that of the ethyl pyridine phase, but with a shorter runtime. The separation using phenyl phase, which had the poorest selectivity under these conditions, could not improved with further reduction in modifier or increase in temperature. The piNap column shows excellent separation of cholesterol metabolites with the most structural similarity, but could not resolve 7-dehydrochesterol from cholesterol. The morpholine phase did not separate the critical pair cholesterol and lathosterol.

Figure 4: Linearity of the four sterol compounds on the COSMOSIL Quinoline column



The linearity for the four compounds was determined from 2.5 ng to 5 ug on column and Figure 4 shows the overlay TIC chromatograms.

Figure 5 : Chromatogram of the four sterols on a 2.0x150mm COSMOSIL Quinoline Column. The concentration of sample on column was 2.5 ng.



Cosmosil Quinoline column, 2.0x150mm
2% MeOH at 2.5ml/min
Outlet pressure: 127 bar
APCI (+)
5-µL injection

1. 7-Dehydrocholesterol
2. Cholesterol
3. 5 α -Cholest-7-en-3 β -ol
4. 5 α -Cholestan-3-one

Many applications, especially those that have limited sample, require increased sensitivity for the analysis. In addition, the large number of samples required to be run for each time point of a study drives the need for reduced runtimes. As demonstrated in Figure 5, a 2.0mm x 150 mm Cosmosil Quinoline column was used to reduce the runtime to < 2.5 minutes with a 20% increase in sensitivity.

Conclusions

SFC/MS combined with the novel COSMOSIL Quinoline phase has been demonstrated as a viable approach for the analysis of polar lipids. The COSMOSIL Quinoline offers reproducible results with increased selectivity and reduced run time. The COSMOSIL piNap column also offers unique selectivity and can offer enhanced selectivity for the analysis of lipids over convention pi-pi interaction columns.

References

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