LC/ELSD and LC/MS/MS of Cholesterol and Related Sterols on a Poroshell 120 Column

Application Note

BioPharma

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Introduction

Cholesterol and its metabolites, as well as several phytosterols, are separated using Agilent Poroshell 120 columns with LC/ELSD and LC/MS/MS. Two phases of Poroshell 120, EC-C18 and SB-C18, are compared for the sterol separation. Poroshell 120 EC-C18 has better selectivity for these sterols and gives better resolution. LC/MS/MS separates closely eluting compounds, such as lathosterol and cholesterol.

Agilent Technologies

Introduction

The analysis of cholesterol and related compounds can be done by GC and LC. LC methods have several advantages in that they do not require derivatization of sterols prior to analysis, which simplifies the procedure by reducing time spent preparing samples. In addition, LC columns can better withstand the "dirty samples" in which cholesterol is naturally found, such as blood plasma.

Agilent Poroshell 120 columns were selected for this analysis because they can deliver UHPLC efficiencies and throughput with a 2.7 µm particle, resulting in relatively low backpressure. In addition, the 2 µm frit on the Poroshell 120 column is less likely to plug when compared to the 0.5 µm frit typically used on sub 2-µm UHPLC columns, making the Poroshell 120 columns a more rugged column choice for dirty samples.

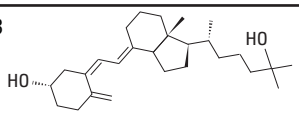
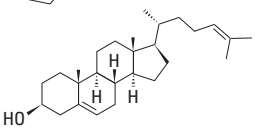
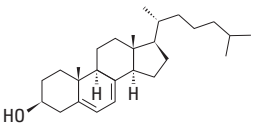
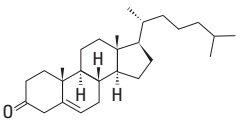
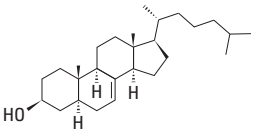
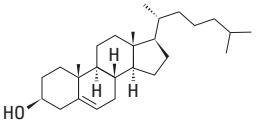
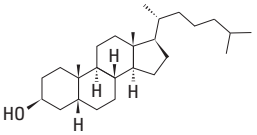
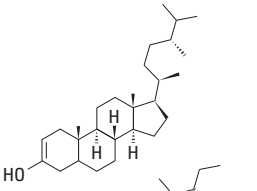
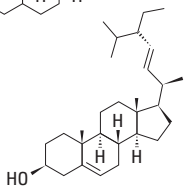
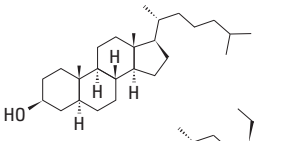
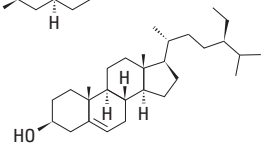
Detection of sterols using LC can be by MS, UV, or ELSD (evaporative light scattering detection). The choice of detector is influenced by the sensitivity needed in the separation, with LC/MS/MS providing very high sensitivity. We chose to develop separations using both ELSD and MS with an Agilent 6460A Triple Quadrupole LC/MS using APCI in positive ion mode [1]. Lathosterol and cholesterol are the most challenging compounds in this separation for 2 reasons. They are difficult to resolve completely, and they need to be resolved for MS/MS detection as they are isobaric. In addition, in a plasma sample, cholesterol is present at a much higher concentration than lathosterol. We selected atmospheric pressure chemical ionization (APCI) in positive ion mode for detection due to the nonpolar nature of sterol compounds. APCI provides good sensitivity for the compounds without derivatization.

Experimental

An Agilent 1200 Infinity Series LC system was used, comprising a binary pump (G1312B), a thermostatted column compartment SL (G1316B), a high performance autosampler (G1367D), and an Agilent LT-ELSD or Agilent 6460A Triple Quadrupole as the detector.

The columns used in the application were:

- Agilent Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)
- Agilent Poroshell 120 EC-C18, 4.6 × 75 mm, 2.7 µm (p/n 697975-902)
- Agilent Poroshell 120 SB-C18, 4.6 × 75 mm, 2.7 µm (p/n 687975-902)

No.	Compound	CAS No.	Structure
1	Calcifediol	63283-36-3	
2	Desmosterol	313-04-2	
3	Provitamin D3	434-16-2	
4	5-Cholesten-3-one	601-54-7	
5	Lathosterol	80-99-9	
6	Cholesterol	57-88-5	
7	Coprostanol	360-68-9	
8	Campesterol	474-62-4	
9	Stigmasterol	83-48-7	
10	Cholestanol	80-97-7	
11	Sitosterol	64997-52-0	

Results and Discussion

In total, 11 sterols were separated on Poroshell 120 EC-C18 and SB-C18 columns using the ELSD. The ELSD is an excellent detector if sensitivity is not a critical issue and is ideal for many compounds when a UV detector is not appropriate due to the lack of chromophores in the analytes. The separation was done using a simple isocratic mobile phase at a controlled temperature of 20 °C for better resolution. Chromatograms in Figure 1 show better resolution on the Poroshell 120 EC-C18 than on the Poroshell 120 SB-C18.

It is noticeable that the challenging compounds lathosterol and cholesterol are completely resolved on the Poroshell 120 EC-C18 but not on the SB-C18. The well end-capped EC-C18 provides more retention for the nonpolar sterols than the SB-C18, which is not end-capped. These 2 different phases provide slightly different selectivity, which may be very significant for the separation.

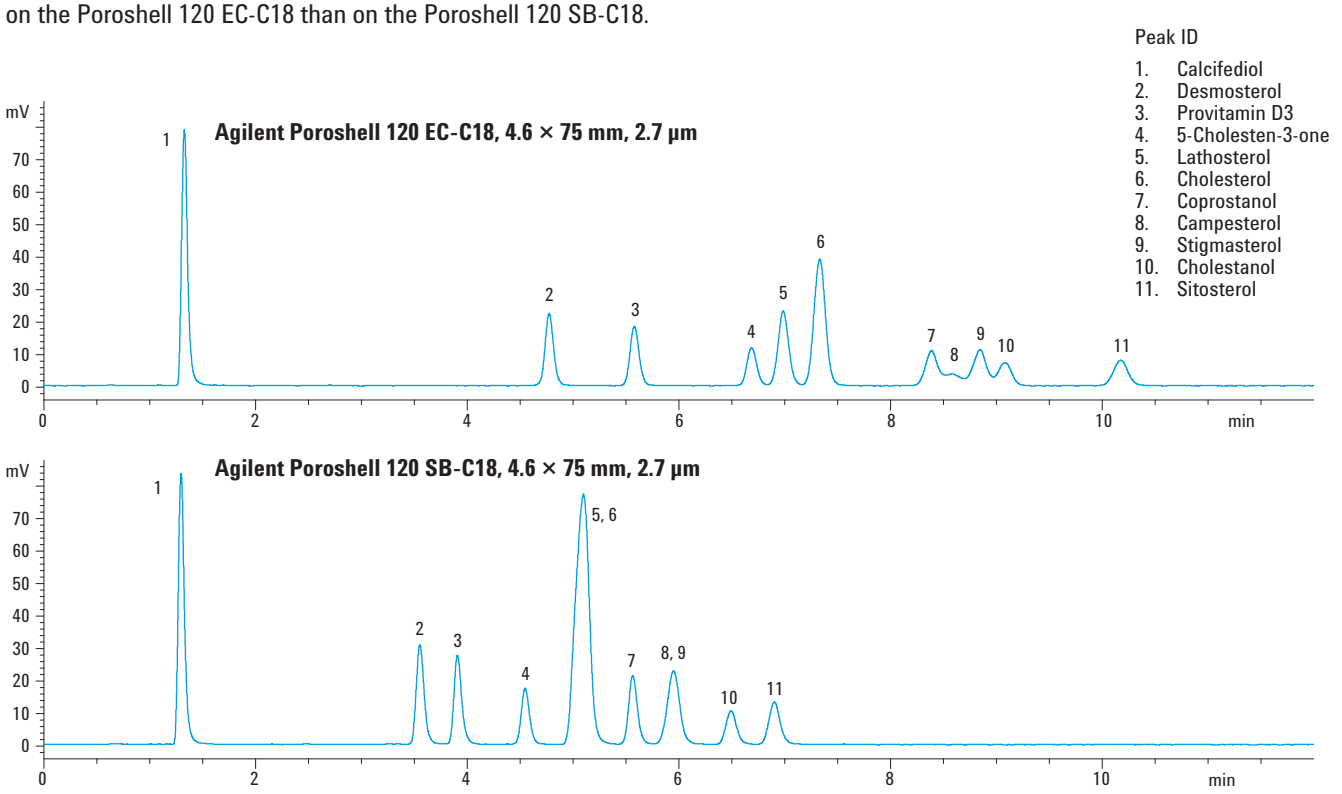


Figure 1. Comparison of Agilent Poroshell 120 bonded phases for the separation of cholesterol and other sterols with evaporative light scattering detection.

Conditions

Sample:	5 µL injection of 11 sterols in methanol
Mobile phase:	80% Acetonitrile:20% methanol
Flow rate:	1.5 mL/min
Temperature:	20 °C
Detector:	ELSD, gain = 8, filter = 3 s, evaporation temperature 60 °C

In a real plasma sample, cholesterol is present at a much higher concentration than other sterols. The ratio of cholesterol to lathosterol is around 2000:1 in a plasma sample, and so it is very difficult for lathosterol to separate from a high concentration of cholesterol. Figure 2 shows a chromatogram of 11 sterols at 2000:1 for cholesterol:other sterols. To achieve sufficient sensitivity for all the sterols with ELSD, 2 measures were adopted. First, a concentration of 10 ppb for low level sterols was needed with ELSD. Second, we chose a 3.0 mm id column that gave higher sensitivity. Though these measures helped to increase sensitivity of low level sterols and thus decrease the concentration of the mixture, cholesterol still had a very high concentration of 20 ppm. With such a high concentration of cholesterol, the Poroshell EC-C18 was overloaded and gave lower resolution with lathosterol.

LC/MS/MS is the better detector choice if additional sensitivity is needed. However, because lathosterol and cholesterol are isobaric, resolution was still required and so the separation was done on the same Poroshell 120 EC-C18, 3.0 × 10 mm, 2.7 µm column using a 6460A Triple Quadrupole LC/MS for the detector. The ratio of cholesterol to lathosterol was still 2000:1 to evaluate the separation of the sterols as they might occur in a plasma sample. However, the mixture was diluted by 1/10 with methanol to give 2 ppm cholesterol and 1 ppb of other sterols. A small injection volume of 2 µL was sufficient for triple quadrupole detection.

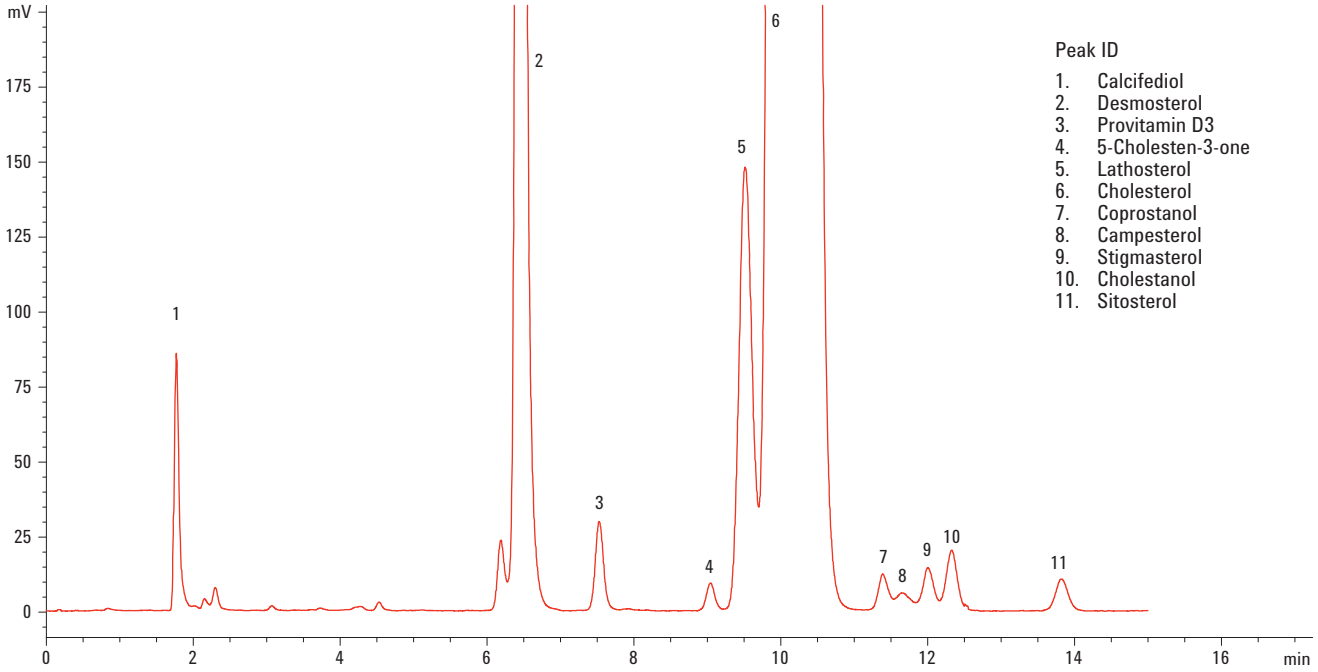


Figure 2. Separation of the sterol mixture (the ratio of cholesterol to other sterols was 2000:1) on an Agilent Poroshell 120 EC-C18, 3 × 100 mm, 2.7 µm column with evaporative light scattering detection.

Conditions

Sample:	5 µL injection of 11 sterols in IPA (20 ppm for cholesterol, 0.01 ppm for other sterols)
Mobile phase:	80% Acetonitrile:20% methanol
Flow rate:	0.6 mL/min
Temperature:	20 °C
Detector:	ELSD, gain = 10, filter = 3 s, evaporation temperature 60 °C

Figure 3 shows that adequate resolution was obtained in this separation using the Poroshell 120 EC-C18 column, even at 2000:1 for cholesterol:lathosterol, and the separation of all compounds was achieved in less than 15 minutes. Because this was an isocratic separation, no additional time was spent on column re-equilibration. This was an efficient and effective method for the separation of these 9 sterols.

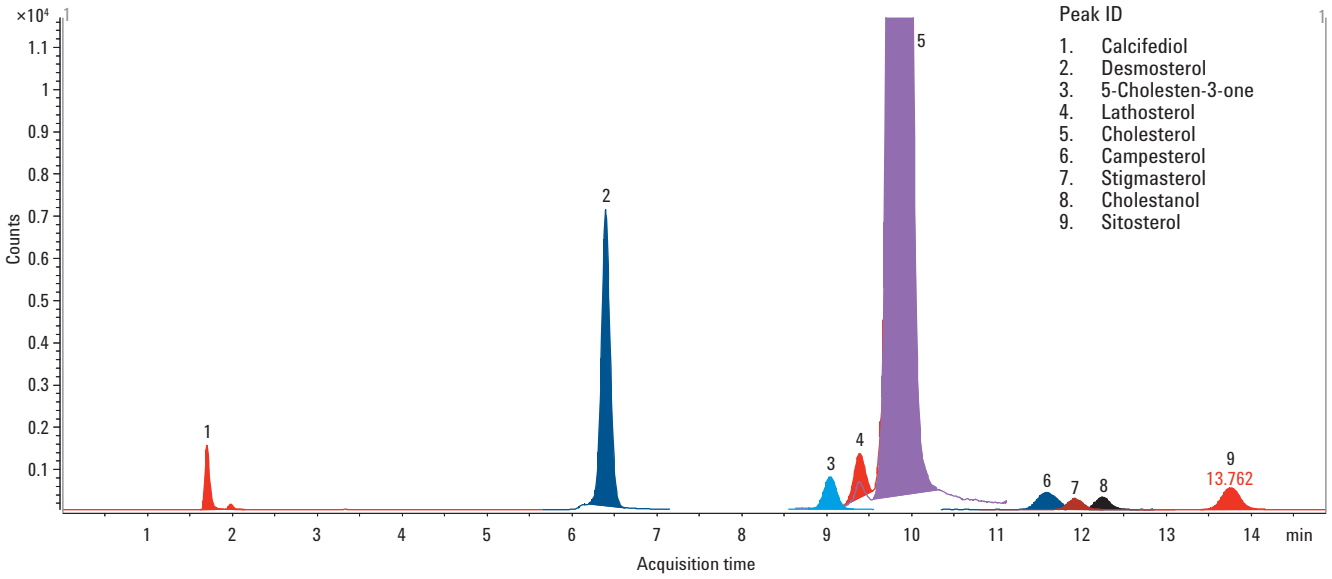


Figure 3. Separation of 9 sterols (the ratio of cholesterol to other sterols was 2000:1) on an Agilent Poroshell 120 EC-C18, 3 × 100 mm, 2.7 µm with LC/MS/MS.

Conditions

Sample:	2 µL injection of 11 sterols in methanol (2 ppm for cholesterol, 1 ppb for other sterols)
Mobile phase:	80% Acetonitrile:20% methanol
Flow rate:	0.6 mL/min
Temperature:	20 °C

MS source parameters

Gas temp:	325 °C
Vaporizer:	350 °C
Gas flow:	4 L/min
Nebulizer:	60 psi
Positive capillary:	4000 V
Corona current:	4 µA

APCI acquisition parameters and transitions

Compound name	Precursor ion	Product ion	Fragmentor	Collision energy (eV)	Ret time (min)
Calcifediol	383.3	211.2	144	25	1.7
Calcifediol	383.3	107.1	144	25	1.7
Desmosterol	367.3	161.1	100	17	6.4
Desmosterol	367.3	95	100	22	6.4
5-Cholesten-3-one	385.4	109.1	128	40	9.05
5-Cholesten-3-one	385.4	97	128	21	9.05
Lathosterol	369.4	95.1	112	29	9.4
Lathosterol	369.4	81.1	112	40	9.4
Cholesterol	369.4	161.2	166	10	9.87
Cholesterol	369.4	95.2	166	38	9.87
Campesterol	383.4	161.2	142	16	11.6
Campesterol	383.4	95	142	30	11.6
Stigmasterol	395.4	83.1	148	17	11.95
Stigmasterol	395.4	81.1	148	37	11.95
Cholestanol	371.4	149	150	15	12.27
Cholestanol	371.4	95	150	30	12.27
Sitosterol	397.4	161	125	18	13.78
Sitosterol	397.4	135.2	125	12	13.78

Conclusions

The separation of cholesterol, some of its metabolites, and other phytosterols was most effectively performed with an Agilent Poroshell 120 EC-C18 column with APCI detection in the positive ion mode on an Agilent 6460A Triple Quadrupole LC/MS detector. This column provided good resolution between cholesterol and lathosterol, an indicator of cholesterol synthesis in the body, even at a ratio of 2000:1. This was critical as the 2 compounds had the same molecular weight and resolution was needed to effectively quantitate these 2 analytes.

Reference

1. N. Kornél, A. Jakab, F. Pollreisz, D. Bongiorno, L. Ceraulo, M. R. Averna, D. Noto, K. Vékey. *Rapid Commun. Mass Sp.* 20, 2433 (2006).

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