Fast Screening Methods for Steroids by HPLC with Agilent Poroshell 120 Columns

Application Note

Pharma, BioPharma, and Clinical Research

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Introduction

Steroids are a type of lipid derived from cholesterol. The main feature of steroids is the ring system of 3 cyclohexanes and 1 cyclopentane in a fused ring system, as shown in Figure 1. There are a variety of functional groups that may be attached. The main feature, as in all lipids, is the large number of carbon-hydrogens, which makes steroids non-polar [1].

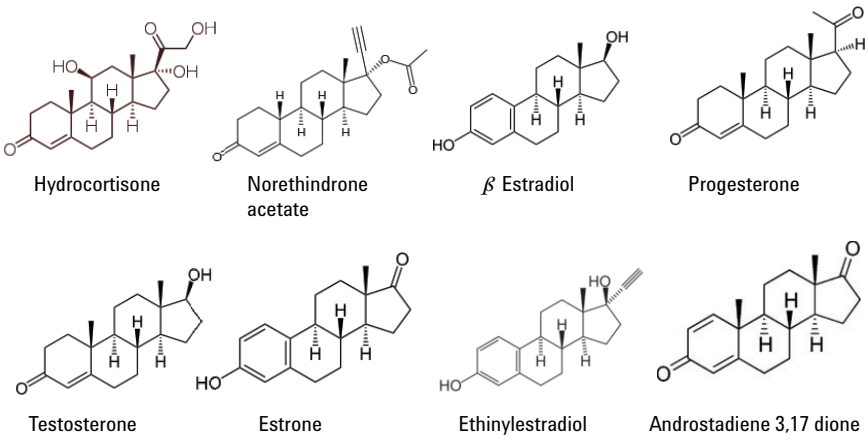

Figure 1. Structures of selected steroids.

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Using Selectivity to Enhance Separation of Steroids

When considering the best way to increase chromatographic resolution, it can be useful to consider the resolution equation, which relates efficiency, selectivity, and retention faction.

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{1 + k'_B}{k'_B}\right)$$

To obtain high resolution, the 3 terms must be maximized. An increase in N, the number of theoretical plates, by lengthening the column, leads to an increase in retention time and increased band broadening. This may not be desirable. Instead, to increase the number of plates, the height equivalent to a theoretical plate can be reduced by reducing the particle size of the stationary phase particles. Superficially porous particles, such as Agilent Poroshell 120, achieve 90% of the efficiency of 1.8 µm materials with considerably lower pressure.

The selectivity factor, α, can also be manipulated to improve separations. Changing selectivity is the variable that can have the largest impact on any separation. Selectivity can be increased by:

- Changing mobile phase composition
- Changing column temperature
- Changing composition of stationary phase

Selectivity is the most powerful tool to optimize separations in HPLC. This parameter is changed by using different bonded phases, including C18, C8, polar embedded, and phenyl bonded phases, or by changing the mobile phase. In this work, Poroshell 120 columns and the Agilent 1200 SL Method Development Solution were used to quickly evaluate method development choices for the analysis of steroids. The short column length and high efficiency provided short analysis times and rapid equilibration leading to fast investigations of selectivity.

Experimental

The Agilent 1260 Infinity Series LC Multi-Method Solution was used. This system consisted of:

- 1260 Infinity Binary Pump (G1312B)
- 1290 Infinity Thermostatted Column Compartment (G1316C)
- 1260 Infinity High Performance Autosampler (G1367E)
- 1290 Infinity Diode-Array Detector (G4212A), equipped with 10 mm MaxiLight cartridge flow cell
- G6140 Single Quadrupole Mass Spectrometer.

The Agilent 1260 Infinity Series LC Multi-Method Solution is a highly flexible system that can be used for up to 4 (100 mm) columns. In addition, the Agilent ChemStation Method Scouting Wizard automates the setup of methods and sequences to screen the available combinations of columns, solvents, predefined gradients, and temperatures. In this work, 4 Agilent Poroshell 120 columns were used:

- Agilent Poroshell 120 StableBond SB-C18, 2.1 × 100 mm, 2.7 µm (p/n 685775-902)
- Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-902)
- Agilent Poroshell 120 Bonus-RP, 2.1 × 100 mm, 2.7 µm (p/n 685775-901)
- Agilent Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 2.7 µm (p/n 695775-912)

The TCC was fitted with a 6 position/6 port selection valve. This is a new Quick Change Valve mounted on a slide-out rail to make plumbing and maintenance more convenient. Port 1 was connected to a StableBond C18 column, and port 2 was connected to an EC-C18 column. Port 3 was connected to a Bonus-RP column, port 4 to a Phenyl-Hexyl column, and port 6 to a bypass connecting capillary.

The solvent passing into each column was heated using 1 of 4 individual low-dispersion heat exchangers. A G1160 12 solvent selection valve was connected to valve position A1 on the G1312B. Together with the internal solvent selection valve of the Binary SL Pump, up to 15 solvents could be screened using this system. The mobile phase was methanol or acetonitrile with 0.1% formic acid and water with 0.1% formic acid. An acetonitrile/water mixture (50%/50% v/v) was used to rinse the modifiers from the columns and allow proper column storage. Agilent ChemStation version B.04.02 was used to control the instrument and process the data.

The compounds examined included hydrocortisone, norethindrone acetate, estradiol, progesterone, testosterone, estrone, ethinylestradiol, and boldione, which were all purchased from Sigma Aldrich. Structures and details are shown in Figure 1 and Table 1. All samples were prepared at 10 mg/mL in acetonitrile and were diluted in water to a final concentration of 0.1 mg/mL.

Column choice to enhance selectivity

The columns were chosen to improve selectivity in the separation. They included a highly end capped column recommended as a first choice in method development (Poroshell 120 EC- C18), and a non end capped C18 (Poroshell 120 StableBond SB-C18) that could have interaction with silanol groups to provide an alternative C18 selectivity using neutral to low pH mobile phases. A polar-embedded amine column (Poroshell 120 Bonus-RP) and a phenyl-hexyl column (Poroshell 120 Phenyl-Hexyl) were also used. Phenyl bonded phases are known for their improved selectivity for aromatic compounds.

A polar-embedded group inserted into the hydrophobic C14 alkyl chain allows the Bonus-RP phase on totally porous Poroshell 120 to minimize interaction of polar samples with silanols, providing symmetrical peaks for a wide variety of applications. This phase is especially useful at neutral pH where amines can interact strongly with ionized silanols. The polar-embedded group also helps to wet the hydrophobic chains and prevents phase collapse in highly aqueous mobile phases.

Poroshell 120 Bonus-RP can be used for many of the same separations as a C18 column while avoiding some of the disadvantages of C18, such as poor wettability in high aqueous mobile phases. In addition, it is much more retentive for those molecules that can interact by hydrophobic interactions and also by H-bonding with the amide group. Compared to alkyl only phases, Bonus-RP has enhanced retention and selectivity for phenols, organic acids, and other polar solutes due to strong H-bonding between polar group (H-bond acceptor) and H-bond donors, like phenols and acids. Bonus-RP gives retention slightly less than a C18 allows, for easy column comparison without the need to change mobile phase conditions. The Bonus-RP phase gives different selectivity than C18 for polar compounds. It is also compatible with 100% water.

The Phenyl-Hexyl phase has unique reversed-phase selectivity, especially for polar aromatics and heterocyclic compounds, derived from analyte interaction with the aromatic ring of the bonded phase and its delocalized electrons. Poroshell 120 Phenyl-Hexyl can be orthogonal to both C18 and Bonus-RP phases. More retention and selectivity will often be observed for solutes with aromatic electron-withdrawing groups such as fluorine or nitro groups [2,3,4].

Table 1. Steroid nomenclature and molecular characteristics.

Common name	IUPC name	Molecular formula	Molecular weight
Hydrocortisone	Cortisol	$C_{21}H_{30}O_5$	362.460
Norethindrone acetate	(17α)-17-ethynyl-3-oxoestr-4-en-17-yl acetate	$C_{22}H_{28}O_3$	340.456
β Estradiol	(17β)-estra-1,3,5(10)-triene-3,17-diol	$C_{18}H_{24}O_2$	272.38
Progesterone	Pregn-4-ene-3,20-dione	$C_{21}H_{30}O_2$	314.46
Testosterone	(8R,9S,10R,13S,14S,17S)- 17-hydroxy-10,13-dimethyl- 1,2,6,7,8,9,11,12,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one	$C_{19}H_{28}O_2$	288.42
Ethinylestradiol	19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol	$C_{20}H_{24}O_2$	296.403
Androstadiene 3,17 dione (boldione)	(8R,9S,10R,13S,14S)-10,13-dimethyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-dione	$C_{19}H_{24}O_2$	284.39
Estrone	3-hydroxy-13-methyl- 6,7,8,9,11,12,13,14,15,16-decahydrocyclopenta[a]phenanthren-17- one	$C_{18}H_{22}O_2$	270.366

Poroshell 120 Phenyl-Hexyl columns deliver unique selectivity for compounds with aromatic groups, providing superior resolution for these samples. Poroshell 120 Phenyl-Hexyl can also provide optimum separations of moderately polar compounds where typical alkyl phases (C18 and C8) do not provide adequate resolution. Acetonitrile tends to decrease the π–π interactions between aromatic and polarizable analytes and the phenyl-hexyl stationary phases, but methanol enhances those same interactions, giving both increased retention and changes in selectivity [5]. This does not mean that acetonitrile should not be used with a phenyl bonded phase or that it might not provide an acceptable separation, but methanol is more likely to deliver the additional selectivity that is desired from a phenyl phase.

Results and Discussion

As can be seen in Figure 2, the separation of all 8 compounds was attempted on all columns surveyed. The Poroshell 120 EC-C18 and Poroshell 120 Phenyl-Hexyl columns showed very similar profiles, although the elution on the Phenyl Hexyl column was faster. This could indicate that the π–π interactions on the Phenyl-Hexyl column were being reduced by the acetonitrile. The overlap of estradiol and androstadiene was less severe on the Phenyl-Hexyl column. The Poroshell 120 SB-C18 column delivered a very different separation, resolving estradiol but losing resolution on ethinylestradiol and estrone. This could be due to the exposed silanols on the SB-C18 phase or to some additional shape selectivity derived

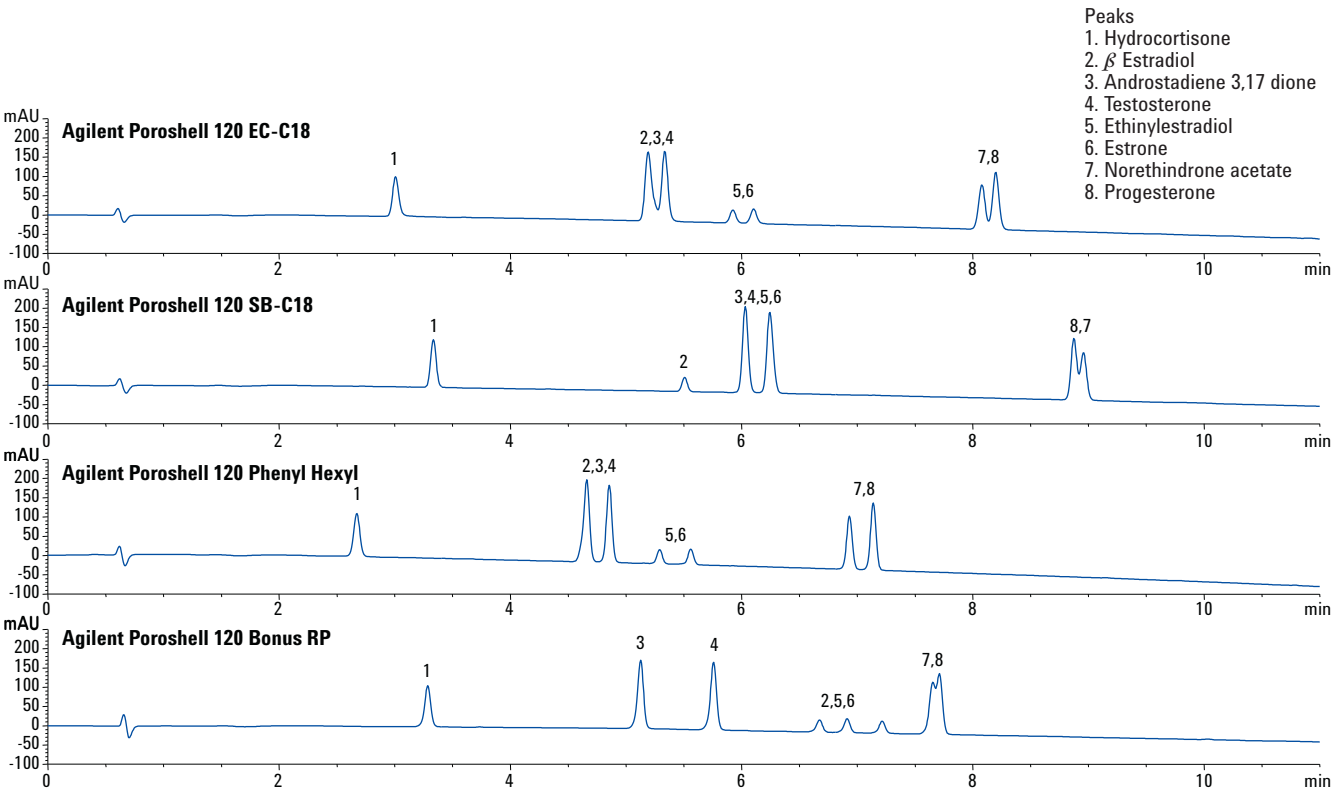

Figure 2. Separation of steroids using Agilent Poroshell 120 columns with acetonitrile.

Conditions

Columns:	Agilent Poroshell 120, 2.1 × 100 mm
Flow rate:	0.4 mL/min
Gradient:	25-80% MeCN/10 min (0.1% formic acid in water and MeCN)
Temperature:	25 °C
Detection:	DAD 260,80 ref = off

from the di-isobutyl side chains on the SB-C18 phase. Some additional work is needed to determine this. The Poroshell 120 Bonus-RP phase almost separates all 8 compounds, and when using acetonitrile, it would provide the best method development option for further development.

In Figure 3, the separation was carried out using methanol at slightly elevated temperature (40 °C). In this case, the 2 C18 phases (Poroshell 120 EC-C18 and Poroshell 120 SB-C18) yielded nearly identical chromatographic profiles. Some additional retention was seen on the SB-C18 phase due to some silanol interaction. The Poroshell 120 Bonus-RP chromatogram had 3 overlapping peak pairs, which would likely make further method development difficult in methanol. However, the Poroshell 120 Phenyl-Hexyl phase resolved 8 compounds at better than baseline resolution.

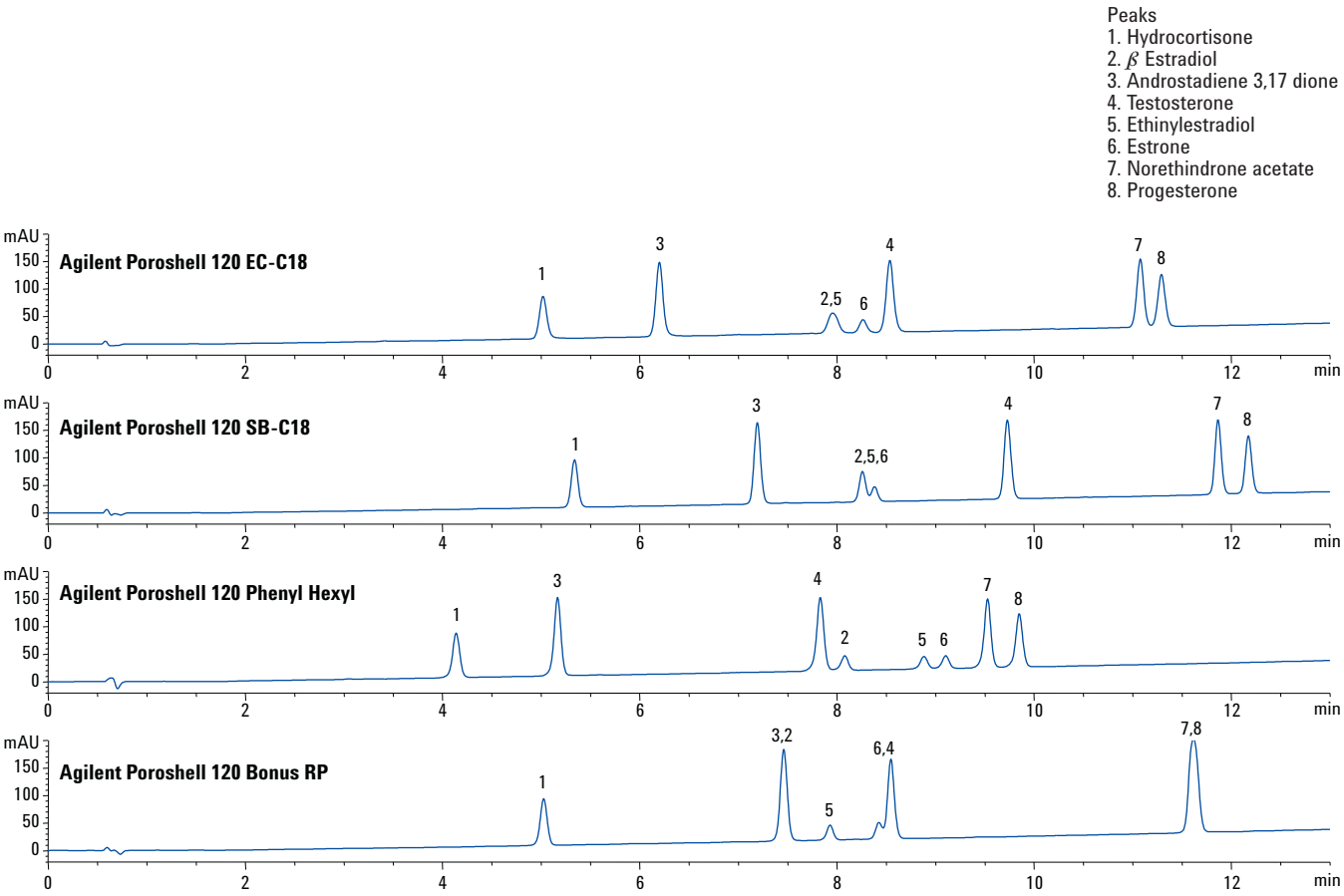

Figure 3. Separation of steroids using Agilent Poroshell 120 columns with methanol.

Conditions

Columns:	Agilent Poroshell 120, 2.1 × 100 mm
Flow rate:	0.4 mL/min
Gradient:	40-80% MeOH/14 min (0.1% formic acid in water and MeOH)
Temperature:	40 °C
Detection:	DAD 260, 80 ref = off

Conclusions

Analysis problems can be quickly resolved by including survey methods with generic gradients as part of the method development scheme. This work used steroids as an example, and showed how phases and organic modifiers, such as acetonitrile and methanol, could develop different selectivity that could be used to optimize the separation. In this case, the widely used C18 phases, as found on Poroshell 120 EC-C18 and SB-C18 columns, did not provide adequate separation. Using an alternative selectivity column such as Poroshell 120 Bonus-RP in acetonitrile or Poroshell 120 Phenyl Hexyl yielded better results, and could be used for several thousand samples.

References

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Printed in the USA
August 2, 2012
5991-0451EN

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