Fast Screening Methods for Analgesics and Non-Steroidal Anti-Inflammatory Drugs by HPLC

Application Note

Small Molecule Pharmaceuticals and Generics

Author

William Long

Agilent Technologies, Inc.

Introduction

Some of the earliest recorded medical treatment involves management of pain with analgesics. Hippocrates first reported using willow bark [1] to mitigate signs of inflammation. On 25 April 1763, Edward Stone wrote to the Royal Society describing his observations on feverish patients of using medicines based on willow bark. The active ingredient of willow bark was first isolated by Johann Andreas Buchner in 1827. By 1829, French chemist Henri Leroux had improved the extraction process, and in 1897 the German chemist Felix Hoffmann began a new age of pharmacology by converting salicylic acid into acetylsalicylic acid - named aspirin. Other NSAIDs Nonsteroidal anti-inflammatory drugs (NSAIDs) were developed from the 1950s onward [2].

NSAIDS are a class of drugs that deliver analgesic and fever-reducing effects, and, in higher doses, anti-inflammatory effects. As analgesics, NSAIDs are unusual in that they are non-narcotic and, thus, are used as a non-addictive alternative to narcotics [3].

The most prominent members of this group of drugs, aspirin, ibuprofen, and naproxen, are all available over the counter in most countries [4]. Acetaminophen and phenacetin are not considered NSAIDs because they have little anti-inflammatory activity.

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Using selectivity to enhance separation of analgesics

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 (Equation 1).

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

Equation 1.

To obtain high resolution, the three terms must be maximized. Lengthening the column to increase N, the number of theoretical plates, leads to an increase in retention time and increased band broadening. This may not be desirable. Instead, the height equivalent to a theoretical plate can be reduced by reducing the 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 the selectivity variable can have the largest impact on any separation. Selectivity can be increased by changing mobile phase composition or by changing the composition of the stationary phase.

Selectivity is, therefore, the most powerful tool to optimize separations in HPLC. This parameter is changed by using different bonded phases, including C18, polar embedded, phenyl, and perfluorophenyl, or by changing the mobile phase. In this application note, 4.6 × 50 mm, 2.7 µm Poroshell 120 columns were used to quickly evaluate method development choices for the analysis of steroids. The short column length and high efficiency of these columns provided short analysis times and rapid equilibration, leading to fast investigations of selectivity.

Materials and Methods

An Agilent 1260 Infinity Binary LC was used, consisting of:

- G1312B Binary Pump SL, capable of delivering up to 600 bar
- G1316C Thermostatted Column Compartment (TCC)
- G1376D High Performance Autosampler SL Plus
- G4212A Diode Array Detector equipped with a G4212-60008 10-mm path length, 1-µL flow cell

The columns used were:

- Agilent Poroshell 120 PFP, 4.6 × 50 mm, 2.7 µm (p/n 699975-408)
- Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm (p/n 699975-902)
- Agilent Poroshell 120 Bonus-RP, 4.6 × 50 mm, 2.7 µm (p/n 699968-901)
- Agilent Poroshell 120 Phenyl-Hexyl, 4.6 × 50 mm, 2.7 µm (p/n 699975-912)

A generic gradient separation was used to evaluate the columns, consisting of ammonium formate (20 mM NH_4HCO_2, pH 3.0) using either methanol or acetonitrile. Agilent ChemStation version C.1.05 was used to control the instrument and process the data.

The compounds examined included acetaminophen, phenacetin, piroxicam, tolmetin, ketoprofen, naproxen, sulindac, diclofenac, and diflunisal, which were all purchased from Sigma-Aldrich Corp. The analgesic materials all possessed a wide variety of functional groups including fluorine (sulindac and diflunisal) and chlorine (diclofenac). The structures of these compounds 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. Ammonium formate and formic acid were also purchased from Sigma-Aldrich. Methanol and acetonitrile were purchase from Honeywell (Burdick and Jackson). Water used in this work was 0.2 µm filtered 18 MΩ from a Milli Q system (Millipore).

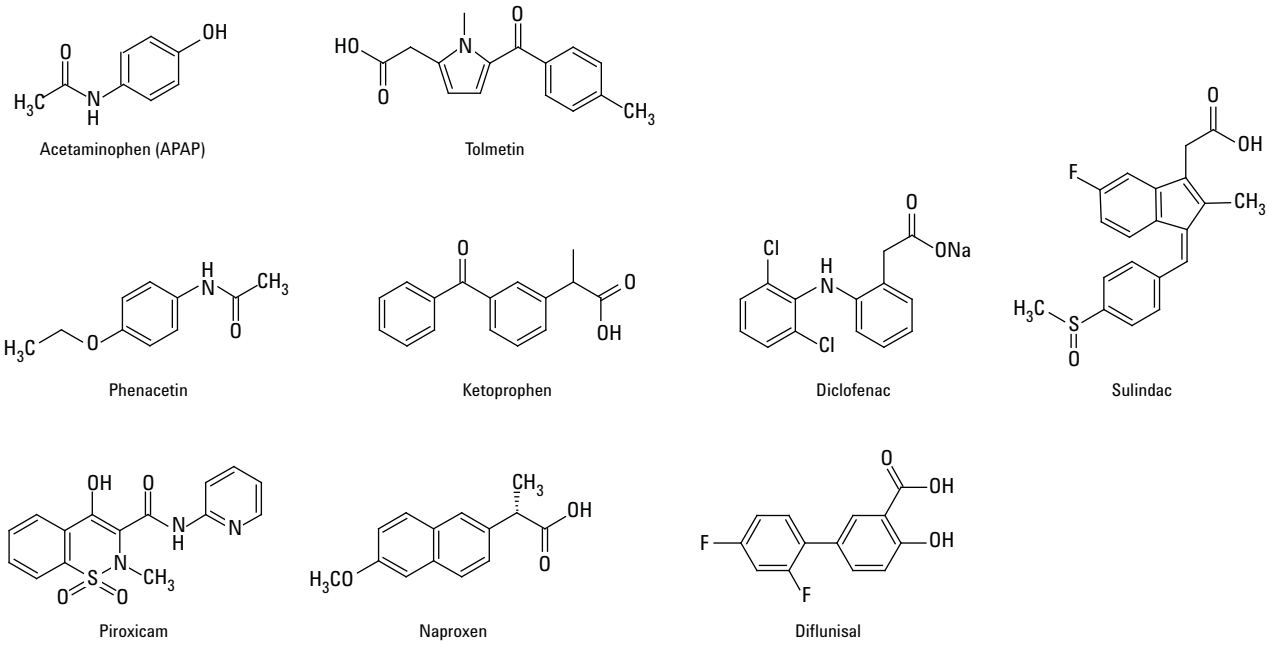

Figure 1. Structures of analgesics.

Table 1. Retention time, log P, and pKa data for analgesics.

Compound	log P	pKa	tr PFP MeCN	tr PFP MeOH	tr C18 MeCN	tr C18 MeOH	tr BrP MeCN	tr BrP MeOH	tr PH MeCN	tr PH MeOH
Acetaminophen	0.46	9.38	0.863	1.252	0.803	1.123	0.99	1.235	0.781	1.039
Phenacetin	1.58	2.2	1.966	2.912	2.147	2.943	2.176	2.774	2.059	2.959
Piroxicam	3.06	6.3	2.536	3.876	2.849	3.688	2.744	3.415	2.732	4.027
Tolemetin	2.79	3.5	2.868	4.137	2.928	4.265	3.173	4.073	2.893	4.395
Ketoprofen	3.12	4.45	3.008	4.258	3.109	4.308	3.342	4.146	3.137	4.468
Naproxen	3.18	4.15	3.112	4.505	3.249	4.436	3.342	4.218	3.167	4.468
Sulindac	3.42	4.7	2.934	4.656	3.249	4.308	3.173	4.288	2.995	4.594
Diclofenac	4.51	4.15	3.53	4.795	3.9	5.046	4.043	4.87	3.711	5.106
Diflunisal	4.41	2.69	3.659	5.094	3.249	4.567	3.867	4.919	3.091	4.559

Column choice to enhance selectivity

The columns were chosen to improve selectivity in the separation. They included Poroshell 120 EC-C18, a highly end-capped C18 column recommended as the first choice in method development.

A 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 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, such as phenols and acids. Bonus-RP provides slightly less retention than a C18 allows, for easy column comparison without the need to change mobile phase conditions. The Bonus-RP phase gives different selectivity to C18 for polar compounds. It is also compatible with 100% water.

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 these same interactions, giving both increased retention and changes in selectivity. 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 different selectivity that is desired from a phenyl phase.

The Poroshell 120 PFP column possesses a pentafluorophenyl ligand. This can provide an orthogonal separation mechanism to traditional reversed-phase columns. By specifically targeting many polar retention mechanisms, PFP phases can separate analytes based on small differences in structure, substitution, and steric access to polar moieties. The resulting selectivity for positional isomers, halogenated compounds, and polar analytes is particularly useful in the analysis of complex mixtures, and small molecule pharmaceuticals.

Results and Discussion

As can be seen in Figure 2, the separation of all nine compounds was attempted on all columns. The Poroshell 120 PFP and Poroshell 120 Bonus-RP columns fully resolved all compounds in the same order, in about 5 minutes using methanol as the organic modifier. The PFP column provides a more even spacing of all the peaks in comparison to the Poroshell 120 Bonus-RP. The Poroshell 120 Phenyl-Hexyl column did not yield the same elution order as the Poroshell 120 PFP column. This meant that the PFP column was not just a stronger phenyl column; other interactions beside π-π and hydrophobic interactions were in play. All four columns eluted acetaminophen (APAP) and phenacetin first. The Poroshell 120 EC-C18 column did not fully separate three compounds (tolmetin, ketoprofen, and sulindac).

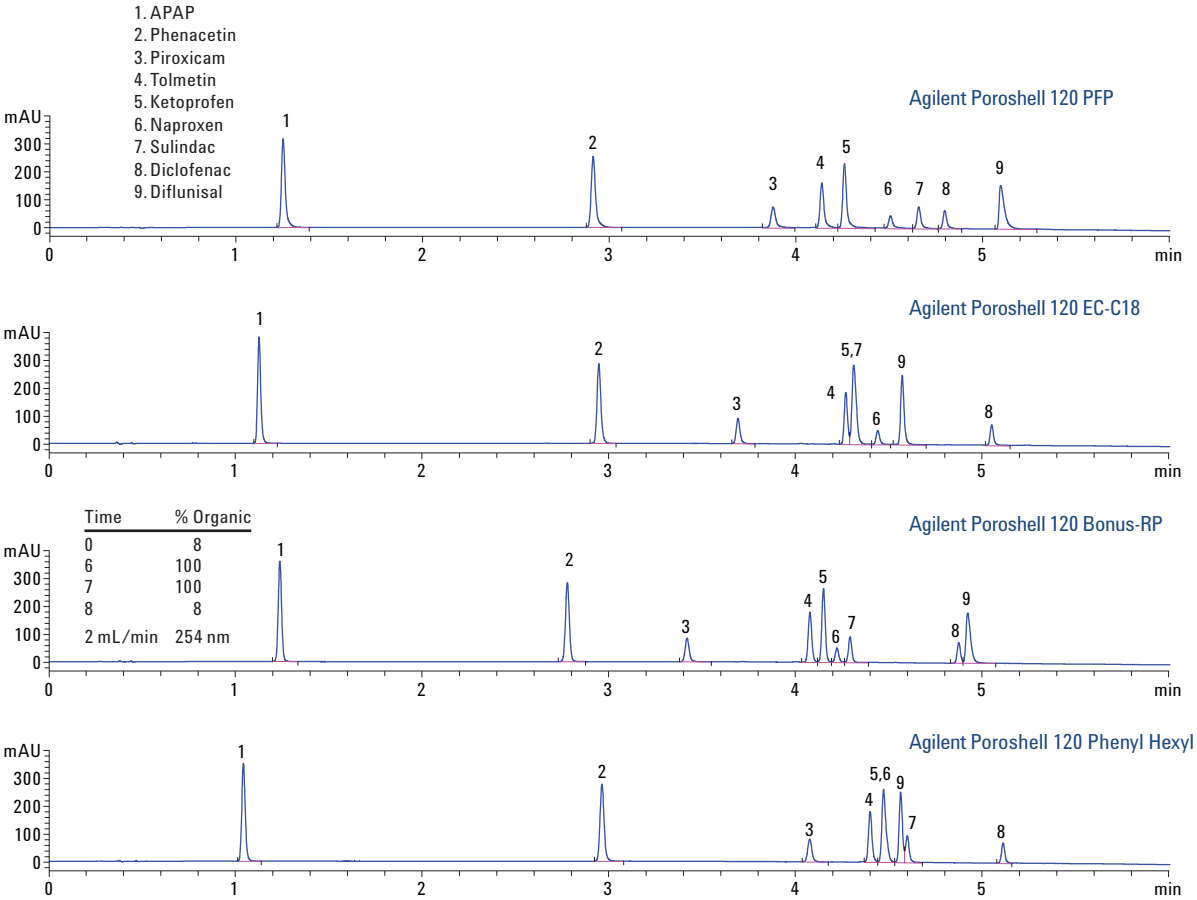

Figure 2. Separation of analgesics using Agilent Poroshell 120 columns using methanol. A) 20 mM NH_4HCO_2, pH 3.0; B) methanol, 40 °C, Agilent 1260 Infinity Binary LC with pulse damper and standard mixing column.

Figure 3 shows the separation on all four columns using acetonitrile. In this case, only Poroshell 120 PFP resolved all compounds. Poroshell 120 EC-C18 and Poroshell Phenyl-Hexyl columns eluted all compounds in the same order. Typically, π-π interactions with Phenyl-Hexyl columns are overwhelmed in acetonitrile. Again, the PFP and Bonus-RP columns had very similar elution orders (with the exception of the last two peaks).

Since the Poroshell 120 PFP phase almost separated all nine compounds, and when using methanol or acetonitrile, it would provide the best method development option for further development.

Table 1 lists the retention times of all nine analytes on the four columns using methanol and acetonitrile. Log P and pKa data are also listed. Log P refers to the equilibrium distribution of a single substance between two solvent phases separated by a boundary.

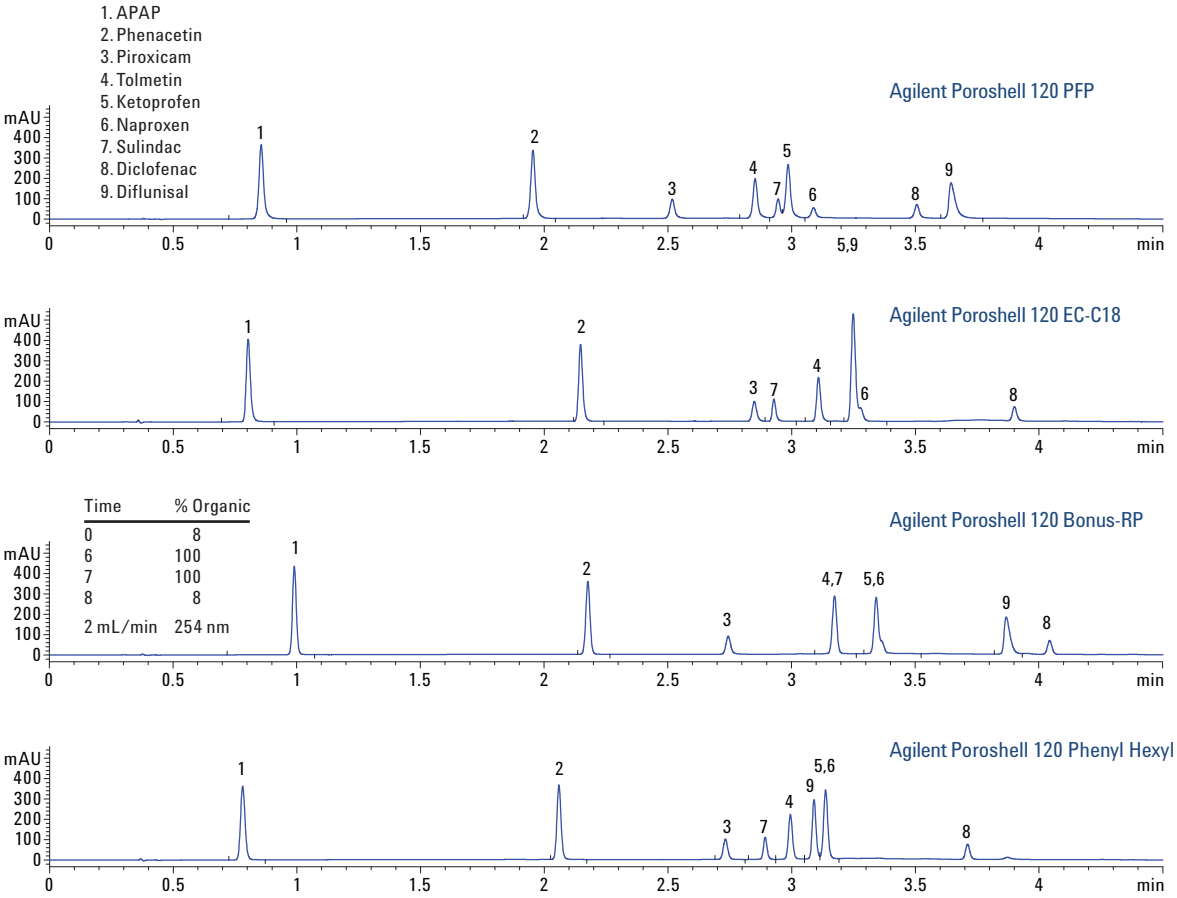

Figure 3. Separation of analgesics using Agilent Poroshell 120 columns with acetonitrile.

It was discovered that the narcotic action of many simple organic solutes was reflected rather closely by their oil-water partition coefficients. The oil was later replaced by octanol [5]. As can be seen in Figure 4, log P values corresponded to retention time on the Poroshell 120 EC-C18 column using methanol or acetonitrile. Figure 5 shows the correlation of Poroshell EC-C18 and PFP retention time data in methanol and acetonitrile for the last seven analgesics. The first of these, acetaminophen and phenacetin, remained in the same elution order in all solvent- column combinations. With these compounds removed the correlation between EC-C18 and PFP retention in methanol (Figure 5a) and acetonitrile (Figure 5b) was poor and highly indicative of orthogonality.

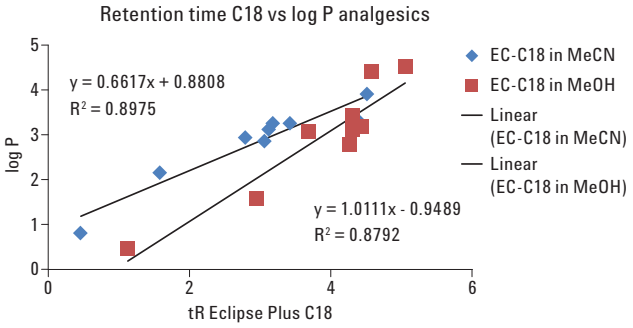

Figure 4. Agilent Poroshell 120 EC-C18 retention time versus log P values.

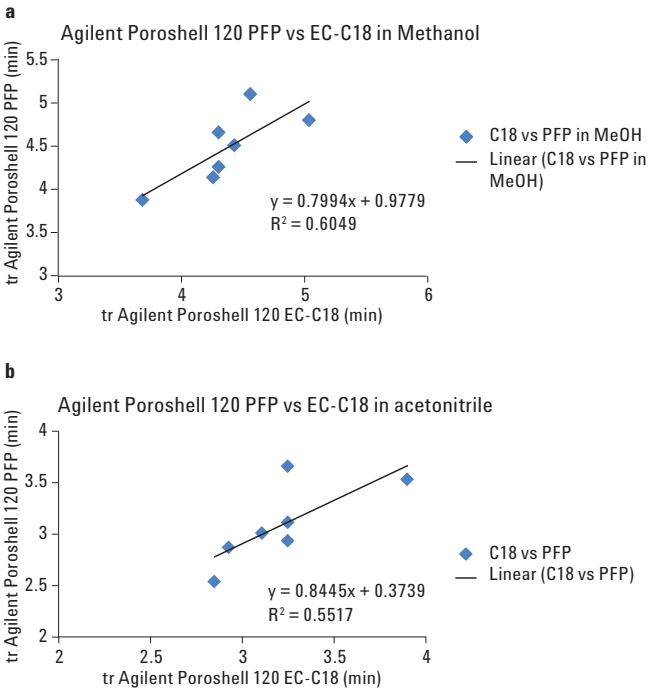

Figure 5. Agilent Poroshell 120 EC-C18 retention time versus Agilent Poroshell 120 PFP retention time in methanol (5a) and acetontrile (5b).

Conclusions

Analysis problems can be quickly resolved by including survey methods with generic gradients as part of the method development scheme. This application note used analgesics as an example, and showed how phases and organic modifiers such as acetonitrile and methanol can develop different selectivity that can be used to optimize the separation. In this case, the widely used C18 phases, as found in Agilent Poroshell 120 EC-C18, did not provide adequate separation. Using an alternative selectivity column such as Agilent Poroshell 120 PFP yielded better results. Fluorinated stationary phases are useful because of their enhanced interaction with halogens, and conjugated compounds.

References

1. N.G. Bisset. *Herbal Drugs and Phytopharmaceuticals*, p. 534. Medpharm Scientific Publishers, Stuttgart, Germany (2004).
2. G. Guerriero. *Ann. NY Acad. Sci*. **1163**, 154 (2009).
3. P. Mackowiak. *Clin. Ther. Dis*. **31**, 154 (2000).
4. A. Streitweiser, K. Heathcock. *Introduction to Organic Chemistry*. MacMillan, NY, USA (1981).
5. http://en.wikipedia.org/wiki/Non-steroidal_anti-inflammatory_drug.
6. J. Sangster. *Logkow: a database of evaluated octanol/water partition coefficients (Log P)*. Sangster Research Laboratories, Montréal, Canada. http://logkow.cisti.nrc.ca/logkow/index.jsp.

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