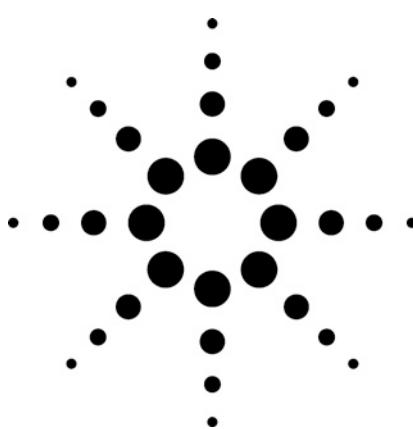# High Throughput HPLC Analysis of Barbiturates

Application Note

Forensic Toxicology

Author

John W. Henderson, Jr., Cliff Woodward, and Ronald E. Majors

Agilent Technologies, Inc.

2850 Centerville Road

Wilmington, DE 19808-1610

USA

Abstract

In this study, an established HPLC method for USP Pheno-barbital was sequentially and quickly improved to a high throughput method using Rapid Resolution (RR) and Rapid Resolution High Throughput (RRHT) techniques. New RRHT column technology is an especially powerful tool for improving lab productivity by reducing HPLC analysis time dramatically. Starting with a proven rugged method, conversion to a high throughput method can be achieved simply by replacing the original analytical-sized column with either a RR or RRHT column. The reasons for this direct conversion include the similar selectivity of smaller 1.8- and 3.5-µm particles compared to larger 5-µm particles, and an engineered particle size distribution that reduces unacceptably high system pressures that may be experienced with other sub-two micron packings.

Introduction

Although hundreds of barbiturates have been synthesized over the past century, only about a dozen are used today. One factor deter-mining which barbiturate is prescribed depends on the duration of its effectiveness.

Duration of effect is determined by chemical structure and this depends mainly on the alkyl groups attached to carbon #5 (see Figure 1) which confer lipid solubility to the drug. The duration of effective action decreases as the total number of carbons on C #5 increases. To be more specific, a long duration effect is achieved by a short chain and/or phenyl group. A short duration effect occurs when there are the many carbons and branches on the alkyl chain [1].

Fast analysis of blood and/or other body fluids or hair can be useful in emergency situations. Figure 1 lists the chemical structures of the barbiturates used in this study.
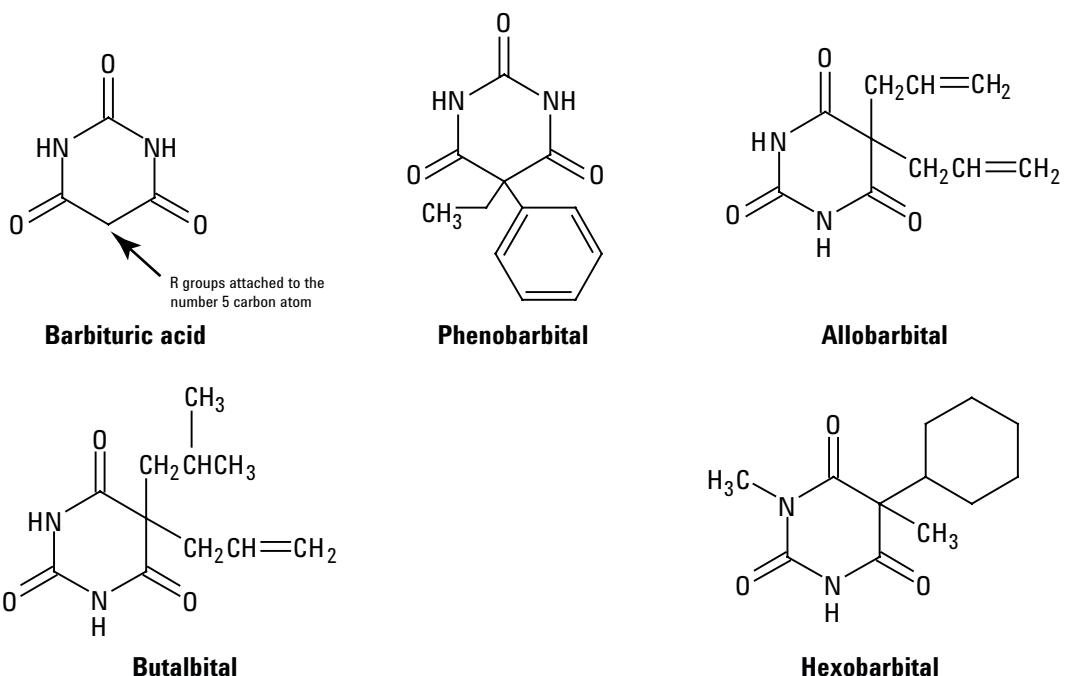
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Figure 1.   Structures of barbiturates used in this study and barbituric acid.

Start with a Proven HPLC Method

The objective of this investigation was to develop a high throughput HPLC method for the analysis of barbiturates based on an existing slower conventional HPLC method. Rapid Resolution (RR) and Rapid Resolution High Throughput (RRHT) HPLC column technology allows one to convert the existing method into a high throughput method easily and straightforwardly to provide a gain in productivity.

For established pharmaceuticals, the starting point for HPLC methods is the United States Pharmacopoeia 27 (USP). The USP method for phenobarbital can be found in reference 3. In addition to the long acting barbiturate, phenobarbital, we added an ultra-short (hexobarbital), a short (allobarbital), and an intermediate (butalbital) acting barbiturate as standards to demonstrate the power of RR and RRHT columns.

Scalability of Barbiturate Reversed-Phase HPLC Method

Scalability refers to the ability of an HPLC method to use columns of different diameters and/or lengths and particle sizes and still maintain the separation characteristics of the method. Earlier, it was demonstrated that ZORBAX columns with smaller particles and shorter lengths provide similar chromatography in a fraction of the time compared with longer columns and larger particles [3].

They maintain column efficiency and resolution because the particle size decreases in proportion to the column length. To demonstrate the scalability of three different column lengths and particle sizes for the test barbiturates, Figure 2 shows an overlay of three chromatograms. The top chromatogram is the original USP method for phenobarbital with the internal standard (caffeine) but with the three additional barbiturates. The USP method specifies a 4.6-mm × 250-mm column with L1 type stationary phase (C18 phase bonded to silica particles) with a minimum resolution of 1.2 [2]. A ZORBAX Eclipse XDB-C18 column with these dimensions and 5 µm particles was selected for this separation and produced an analysis time of about 32 minutes. Simply replacing this column for a shorter one, 4.6-mm × 100-mm with 3.5 µm particles, reduced analysis time by about a factor of 2.5 (or 13 min), or by a factor of five using a 4.6-mm × 50-mm column with 1.8-µm particles (or 7 min). These latter columns provided faster separations with only a small loss of resolution and are referred to as RR and RRHT columns, respectively. These data show that older methods done on larger particle columns can be easily transferred to RR and RRHT columns without sacrificing separation integrity. As can be noted in Figure 2, the resolution of both the 3.5- and the 1.8-µm columns is easily within the minimum specification of the USP method.
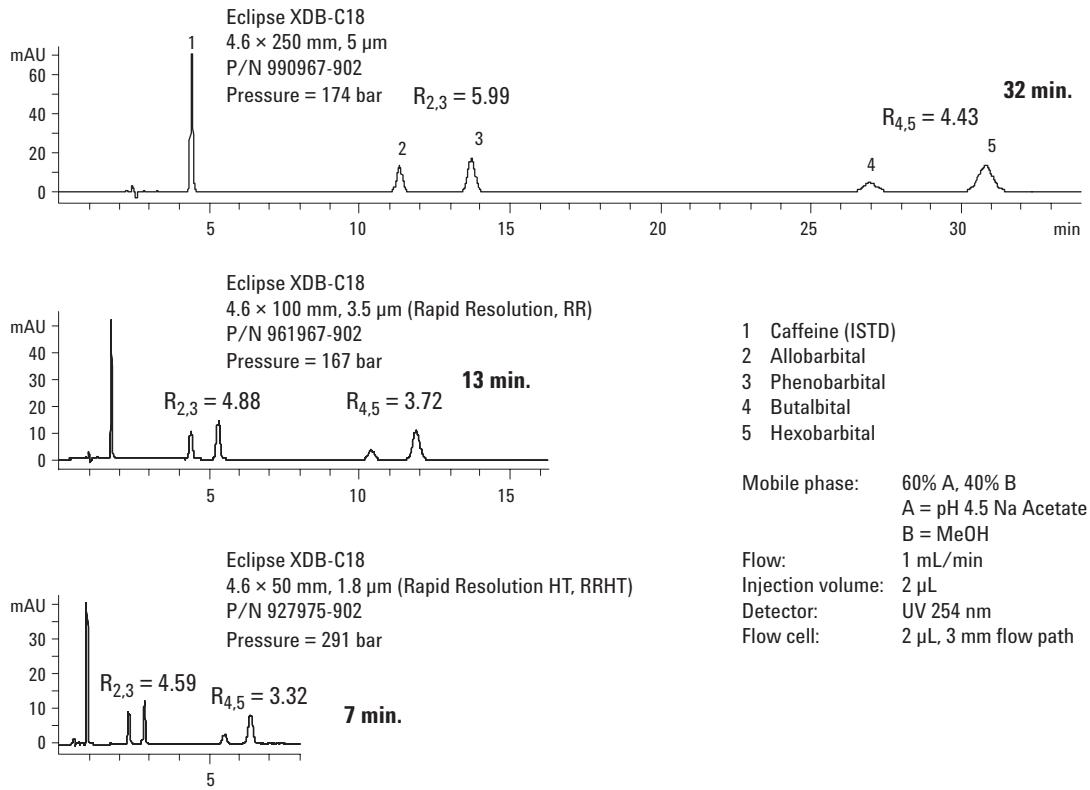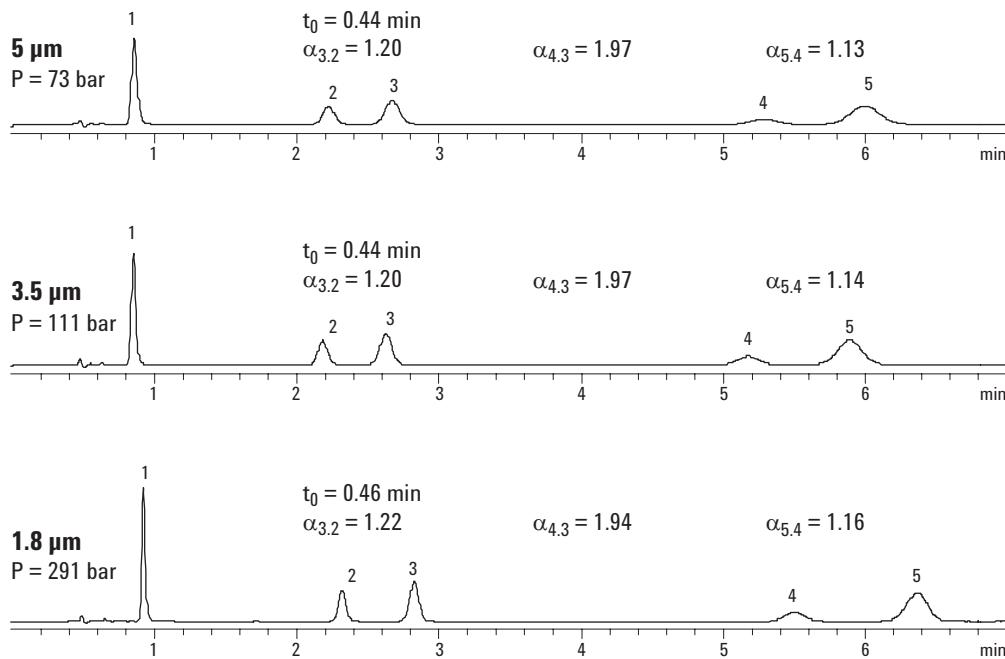
Figure 2.   **RR and RRHT column configurations increase speed and maintain sufficient resolution.**

Stationary Phase Selectivity as a Function of Particle Size

The productivity increase by changing column dimensions and particle size is achieved due to the reproducibility of the spherical silica particles and bonded phase. The highly uniform manufacturing process of the ZORBAX base silica, of the organosilane bonding process, and of the standardized testing of the column leads to the ability of the user to freely substitute columns without suffering selectivity changes which would ultimately affect resolution. One way to measure this uniformity is to compare the selectivity factors among different particle sizes. Selectivity (α) is the ratio of the retention factors: $\alpha_{2,1} = k'_2/k'_1$ where k'_1 is the adjusted capacity factor for compound 1 and k'_2 is the adjusted capacity factor for compound 2.

Figure 3 depicts an overlay of the high throughput separation of barbiturates by equivalent 4.6-mm × 50-mm Eclipse XDB-C18 columns packed with different particle sizes. Note selectivity (α) is the same for all three columns, independent of particle size. The same selectivity indicates the three different sized particles are chemically very similar. The different sized particles can be packed in different column dimensions with predictable and scalable results, because they exhibit the same chromatographic characteristics. Figure 3 also demonstrates that as particle size decreases, efficiency increases. Note that peak widths decrease, producing better sensitivity (taller peaks), as the particle size decreases from 5 to 3.5 to 1.8 µm.

5 µm
P = 73 bar

1

$t_0 = 0.44$ min
$\alpha_{3.2} = 1.20$

$\alpha_{4.3} = 1.97$

$\alpha_{5.4} = 1.13$

2   3

4

5

1   2   3   4   5   6   min

3.5 µm
P = 111 bar

1

$t_0 = 0.44$ min
$\alpha_{3.2} = 1.20$

$\alpha_{4.3} = 1.97$

$\alpha_{5.4} = 1.14$

2   3

4

5

1   2   3   4   5   6   min

1.8 µm
P = 291 bar

1

$t_0 = 0.46$ min
$\alpha_{3.2} = 1.22$

$\alpha_{4.3} = 1.94$

$\alpha_{5.4} = 1.16$

2   3

4

5

1   2   3   4   5   6   min

Columns:	Eclipse XDB-C18, 4.6 x 50 mm
Mobile phase:	60% A, 40% B
	A = pH 4.5 Na Acetate
	B = MeOH
Flow rate:	1 mL/min
Injection volume:	2 µL
Detector:	UV 254 nm
Flow cell:	2 µL, 3 mm flow path

Peak Widths (min)

		5 µm	3.5 µm	1.8 µm
1	Caffeine (ISTD)	0.043	0.033	0.028
2	Allobarbital	0.097	0.073	0.058
3	Phenobarbital	0.116	0.089	0.072
4	Butalbital	0.213	0.165	0.138
5	Hexobarbital	0.237	0.187	0.167

Figure 3.   Particle size influence on selectivity and peak width.

Optimization of Resolution with RRHT technology

A van Deemter plot is often used to depict the changes in column efficiency, usually expressed as H (or Height Equivalent to a Theoretical Plate, HETP), as a function of linear velocity (proportional to flow rate). A typical van Deemter plot is shown in Figure 4a.

The shape of the plot can be described by Equation 1:

$$H = A + B/u + Cu \qquad \text{(Equation 1)}$$

In chromatography, one strives for low values of H that means high values of plates (N). The "A" term in the van Deemter equation, that represents eddy diffusion, is particle size dependent and is minimized for small particles. The "B" term is flow rate dependent and is governed by longitudinal (axial) diffusion. At lower flow velocities, the contribution from axial diffusion to the H value may be quite high and analysis time quite long. The "C" term relates solute mass transfer from the mobile phase to the stationary phase and vice-versa. It is both flow rate and particle size dependent. For a typical column packed with larger particles, say 5- or 10-µm, the column will behave like the van Deemter plot in Figure 4a. However, for smaller particles such as the 1.8-µm packings, the slope of the van Deemter curve at high linear velocities is much flatter, as depicted in Figure 4b. This flatness is due to the superior solute mass transfer into and out of the smaller particles, even at high flow rates. Thus, small particle columns may be run at these higher linear velocities and retain their efficiency while dramatically increasing the separation speed.
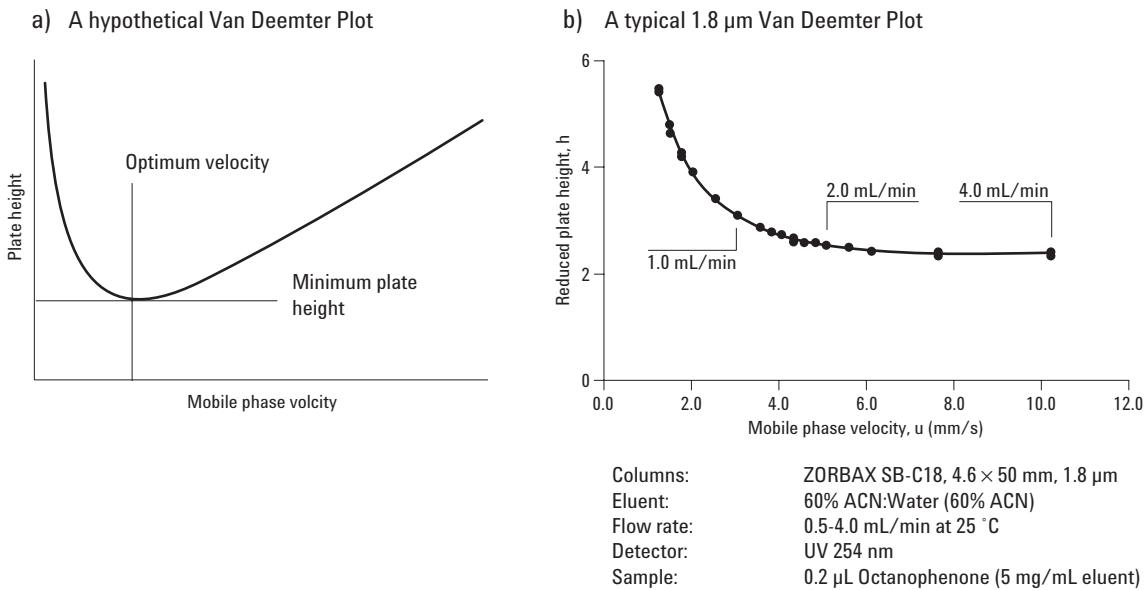
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a) A hypothetical Van Deemter Plot

b) A typical 1.8 μm Van Deemter Plot

Columns:	ZORBAX SB-C18, 4.6 × 50 mm, 1.8 μm
Eluent:	60% ACN:Water (60% ACN)
Flow rate:	0.5-4.0 mL/min at 25 °C
Detector:	UV 254 nm
Sample:	0.2 μL Octanophenone (5 mg/mL eluent)

Figure 4.   Van Deemter plots - plate height versus mobile phase velocity.

Based on the shape of the van Deemter curve in Figure 4b, in order to achieve the best overall efficiency, the 1.8-μm column should be run at higher flow rates (greater than 2-mL/min). Note the comparative chromatograms of Figure 5 where the flow rate was increased from 1-mL/min to 2-mL/min resulting in a more rapid separation and better resolution since the linear velocity was closer to the optimum and column efficiency was greater. Of course, since the column pressure is proportional to the inverse of particle diameter squared, at higher linear velocity the pressure drop will increase, especially for longer columns at room temperature. The column back pressure was 291 bar at 1 mL/min and 550 bar at 2 mL/min. Thus, higher pressure capability for the HPLC instrumentation, such as can be achieved with the Agilent 1200 Rapid Resolution system, is useful. In addition, Agilent's proprietary engineered particle size distribution, unique to the RRHT columns, generates lower system pressure than would be expected for a 1.8-μm particle.
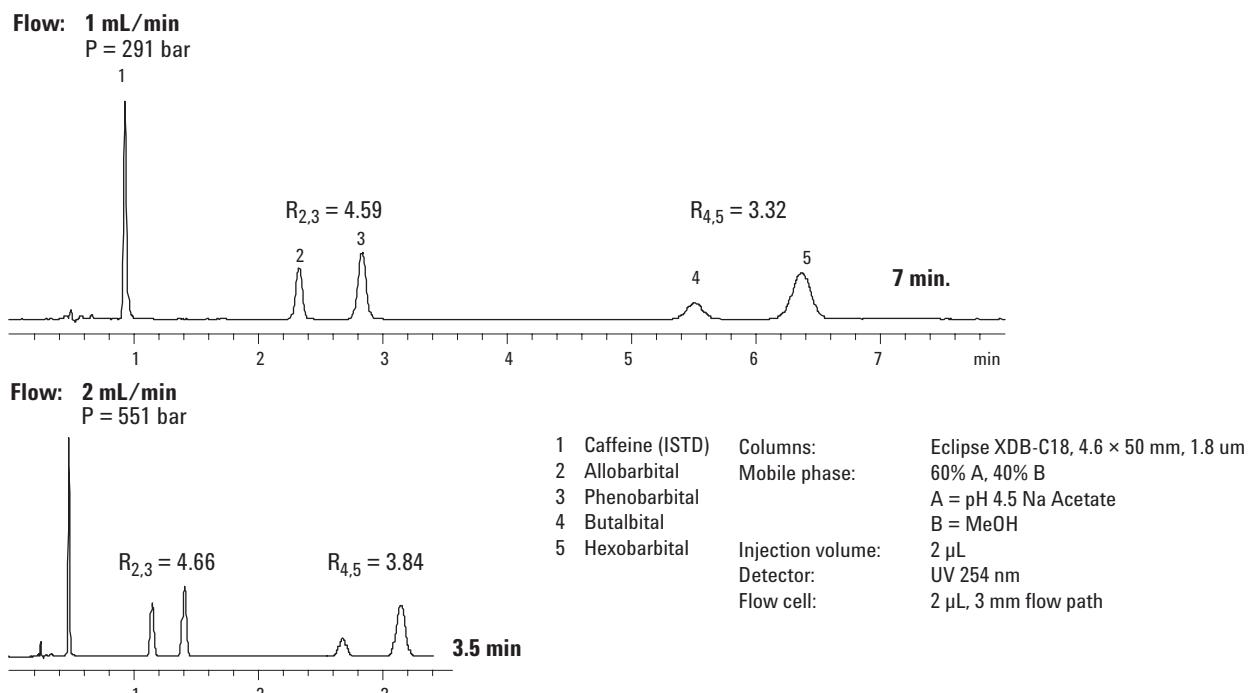

1	Caffeine (ISTD)	Columns:	Eclipse XDB-C18, 4.6 × 50 mm, 1.8 um
2	Allobarbital	Mobile phase:	60% A, 40% B
3	Phenobarbital		A = pH 4.5 Na Acetate
4	Butalbital		B = MeOH
5	Hexobarbital	Injection volume:	2 μL
		Detector:	UV 254 nm
		Flow cell:	2 μL, 3 mm flow path

Figure 5.   Increased flow provides better resolution.

Conclusions

In this study, an established HPLC method for USP Phenobarbital was sequentially and quickly improved to a high throughput method using RR and RRHT techniques. New RRHT column technology is an especially powerful tool for improving lab productivity by reducing HPLC analysis time dramatically. Starting with a proven rugged method, conversion to a high throughput method can be achieved simply by replacing the original analytical -sized column with either a RR or RRHT column. The reasons for this direct conversion include the similar selectivity of smaller particles (1.8- and 3.5-µm) compared to larger 5 µm particles, and an engineered particle size distribution that reduces unacceptably high system pressures that may be experienced with other sub-two micron packings. Additional method development techniques worth considering are use of even faster flow rates, elevated temperature and different bonded phases. The new Agilent 1200 series HPLC system is designed especially for using ZORBAX RRHT columns to take advantage of higher flow rates and elevated temperatures.

References

1. http://www.elmhurst.edu/~chm/vchembook/6673barbit.html

2. USP27-NF22 Page 1461; U.S. Pharmacopeia/National Formulary, 29(6), 2004.

3. J. W. Henderson, Jr., "Plug & Play" Fast and Ultra-Fast Separations Using 3.5-µm Rapid Resolution and 1.8-µm Rapid Resolution High Throughput Columns, Agilent Technologies, publication 5989-2908EN, www.agilent.com/chem

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