

Postcolumn Reactions Enhance Detection Sensitivity and Selectivity in HPLC Analyses

In postcolumn reactions, a chemical reaction occurs after elution of the sample from the column and prior to detection. These reactions can take place without the physical addition of a reagent such as with photochemical irradiation or exposure to an immobilized enzyme or other catalytic reactor. The object of this procedure is to form a molecular species for which the detector has high sensitivity, or to enable the use of more selective conditions (e.g., a different UV wavelength or fluorescence.) Photochemical and enzymatic reactors have been used to generate fluorescent or electrochemically active species. Other reactions can be as simple as altering the pH of the effluent or as complicated as ones involving oxidation and/or hydrolysis prior to derivatization.

Key Words:

postcolumn reaction detection • photochemical reaction • selectivity • sensitivity • PHRED

HPLC analyses of barbiturates can be done rapidly with little sample preparation. Detection, however, presents a set of conflicts. UV detection is commonly used, but in the neutral to acidic conditions needed to prevent damage to silica based columns, barbiturates adsorb UV well only at short wavelengths (200nm or less). By using short wavelength UV, the likelihood of interference from other sample components is increased.

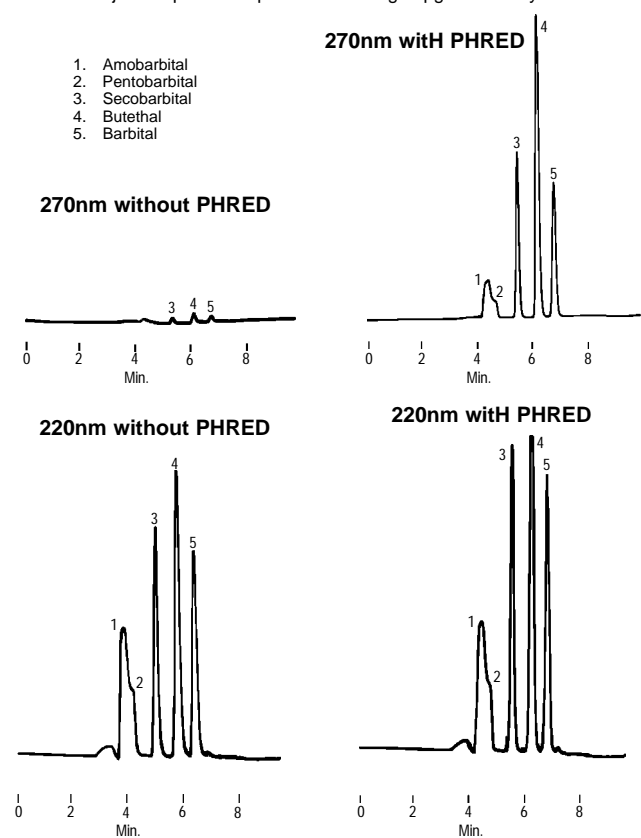
Photochemical reaction has been used to enhance the selectivity and sensitivity for a number of detection schemes, including UV adsorption, fluorescence, electrochemical, and conductivity. The Photochemical Reactor Enhancement Detection System (PHRED) consists of a low pressure mercury lamp with maximum energy at 254nm and various lengths of knitted Teflon® coils which determine the duration of exposure to the radiation. Exposure to UV radiation in the PHRED increases the absorptivity of the barbiturates at higher wavelengths (Figure A). While the absolute signal at 270nm is still lower than that observed at 220nm without radiation, the increased selectivity can allow accurate quantitation when matrix components are not completely resolved (Table 1).

The absorption maximum for barbiturates can be shifted to higher wavelengths by either irradiating the effluent with intense UV light or by raising the pH of the effluent. At alkaline pH, the absorption maxima for barbiturates shifts to longer wavelengths, but high pH mobile phases will dissolve a silica-based packing.

This problem can be solved by raising the pH of the effluent after the barbiturates have been separated and eluted from the column. This is one of the simplest types of postcolumn reactions

Figure A. Barbitals With and Without Photochemical Reactor Enhancement Detection

Column: SUPELCOSIL LC-18, 25cm x 4.6mm ID, 5µm particles
 Cat. No.: 58298
 Mobile Phase: 0.02M NaH₂PO₄, pH 7:acetonitrile, 1:1
 Flow Rate: 1mL/min
 Temp.: ambient
 Det.: UV, 270nm and 220nm
 Reactor Coil: 15m x 0.25mm ID
 Inj.: 10µL mobile phase containing 10µg each analyte



713-0398, 0399, 0400, 0401

Table 1. Enhanced Detection of Barbitals, Using Photochemical Reactor Enhancement Detection

Analyte	Peak Area (x 10 ³)				%Enhancement	
	220nm w/o PHRED	220nm w/ PHRED	270nm w/o PHRED	270nm w/ PHRED	w/PHRED 220nm	270nm
Amobarbital/						
Pentobarbital	202	237	5	171	18	3103
Secobarbital	133	173	3	72	30	2034
Butethal	138	162	3	78	17	2507
Barbital	203	195	4	53	-4	1198

Means for 3 analyses. Column and conditions: see Figure B.

because the reaction time is almost instantaneous and requires only the mixing of a suitable buffer.

The dramatic change in response obtainable is shown in Figure B with quantitative results in Table 2. As little as 2ng of drug can be detected using the post-column reaction system in conjunction with a SUPELCOSIL™ column (5µm packing).

The effect of reagent flow rate is shown in Figure C. Initially there is insufficient reagent and response increases with reagent flow rate. When excess reagent becomes available further increases in reagent flow result in increased dilution and an exponential decrease in response.

The postcolumn reaction system in its simplest form consists of a reagent delivery pump and addition tee (Figure D). Because solutions and solvents do not mix to homogeneity on contact, a mixing device is needed to reduce noise caused by the refractive index effects of mixing occurring in the detector cell. Supelco

Figure B. Postcolumn pH Change Greatly Improves Barbiturates Detection

Column: SUPELCOSIL LC-8, 15cm x 4.6mm ID, 5µm particles
 Cat. No.: 58220
 Mobile Phase: methanol:water, 50:50
 Flow Rate: 1.5mL/min
 Temp.: ambient
 Postcolumn Reagent: 0.05M H₃BO₄, 0.05M NaOH (pH 10.4)
 Reagent Flow Rate: 0.1mL/min
 Mixer: 5cm x 4.6mm column of 75µm glass beads
 Det.: UV, 240nm
 Inj.: 20µL mobile phase containing amounts noted below

1. Barbital, 0.1µg
2. Butethal, 0.1µg
3. Pentobarbital, 0.2µg
4. Secobarbital, 0.1µg

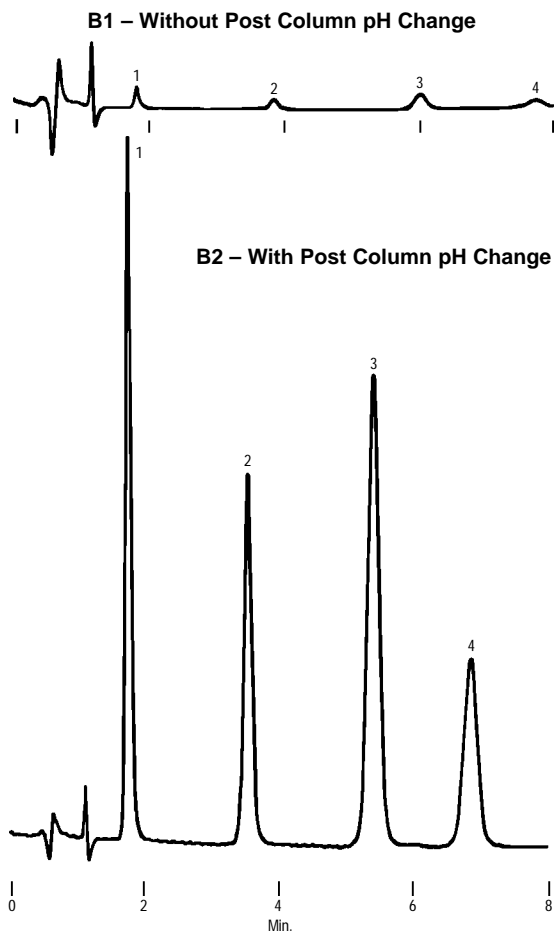


Table 2. Postcolumn pH Change Increases Detection Sensitivity for Barbiturates

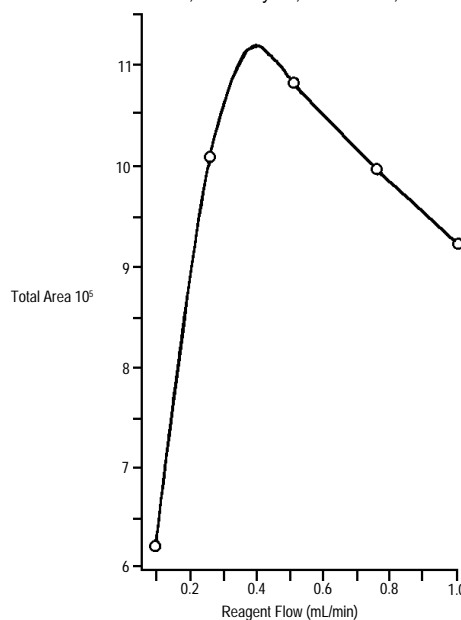
UV Wavelength	Amount of Drug On-Column	Peak Height (mm)*			
		Barbital	Butethal	Pentobarbital	Secobarbital
Without Postcolumn Reaction					
220nm	40ng	15.5	16.0	16.5	7.5
240nm	40ng	11.9	6.5	10.2	5.9
254nm	100ng	13.5	6.9	10.9	5.6
With Postcolumn Reaction					
220nm	4ng	19.7	11.1	15.5	7.9
240nm	2ng	21.6	11.3	14.0	6.3
254nm	20ng	16.6	9.3	12.7	5.8

Conditions as for Figure B, except: Det.: 220/240/254nm UV.

*In all cases, peak to peak noise level was 2mm.

Figure C. Effect of Reagent Flow Rate on Combined Peak Area of Aminoglycoside Antibiotics

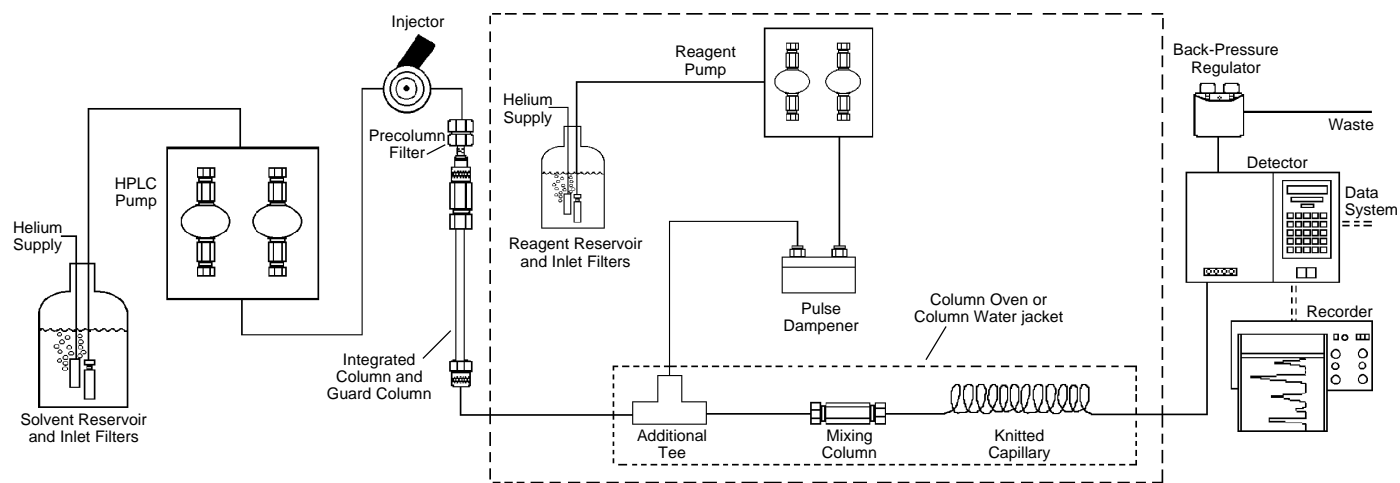
Column: SUPELCOSIL LC-8, 15cm x 4.6mm ID, 5µm particles
 Cat. No.: 58220
 Mobile Phase: tetrahydrofuran:0.01M sodium pentane sulfonate, 0.0056M Na₂SO₄, 0.007M HAc, 2.5:97.5
 Flow Rate: 1.75mL/min
 Temp.: 40°C
 Reagent Flow: 0.1-1mL/min
 Det.: fluorescence, 254nm, Ex., 365nm Em, 600V
 Inj.: 20µL mobile phase containing 0.5µg each of Kanamycin, Amikacin, Tobramycin, Sissomicin, Netilmicin, Neomycin



offers three mixer configurations. They represent different compromises between degree of mixing and the amount of band spreading that is introduced into the system. The 5cm column packed with 75µm glass beads is the best choice for most work. It provides a good level of noise reduction with only moderate loss in peak efficiency. Using larger 250µm glass beads in the 5cm column provides slightly greater noise reduction at the cost of additional dead volume. A third choice is a single bead string reactor which consists of 30cm of 0.5mm Teflon tubing filled with 250µm beads. While this mixer has very low dead volume, it also provides the lowest reduction in noise level.

The fact that a chemical reaction is not instantaneous does not eliminate its use in postcolumn work. The addition of tubing between the point of mixing and the detector allows delay time for the reaction to proceed with increased yield of the detectable

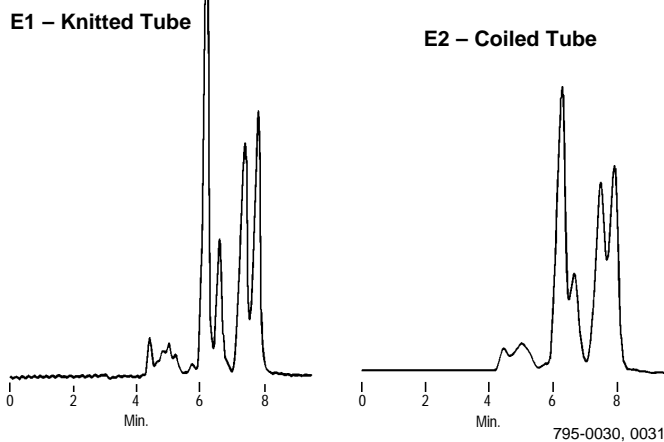
Figure D. HPLC Instrument with Postcolumn Reaction System Components



795-0037

Figure E. A Knitted Capillary Delay Tube Minimizes Band Broadening for Gentamicin Components

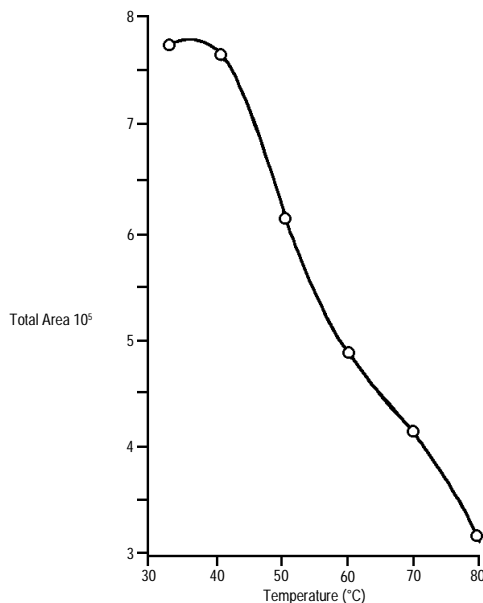
Column: **SUPELCO SIL LC-8-DB, 15cm x 4.6mm, 5µm particles**
 Cat. No.: **58347**
 Mobile Phase: methanol:0.01M sodium pentane sulfonate, 0.0056M Na₂SO₄, 0.007M HAc, 18:82
 Flow Rate: 1mL/min
 Temp.: 50°C
 Reagent: 0.4M H₃BO₃, 0.38M KOH, 6mL/L 40% Brij®-35, 4mL/L mercaptoethanol and 0.8g/L OPA
 Reagent Flow Rate: 0.5mL/min
 Det.: fluorescence, Ex 254nm, Em 365nm, 630V
 Inj.: 20µL mobile phase containing 1.0µg gentamicin



795-0030, 0031

Figure F. Combined Peak Area Is Greatest at Near Ambient Temperature

Column: **SUPELCO SIL LC-8, 15cm x 4.6mm ID, 5µm particles**
 Cat. No.: **58220**
 Mobile Phase: tetrahydrofuran:0.01M sodium pentane sulfonate, 0.0056M Na₂SO₄, 0.007M HAc, 2.5:97.5
 Flow Rate: 1.75mL/min
 Temp.: 30-80°C
 Det.: fluorescence, Ex 254nm, Em 365nm



795-0033

species. While it is known that long lengths of connecting tubing increase band spreading and degrade performance, the amount of band spreading and loss of resolution can be minimized by knitting the tubing. The success of this approach is demonstrated in Figure E, which compares the resolution of gentamicin components with loosely coiled tubing to that obtained with an equal length of knitted tubing.

Reactions can often be accelerated by heating. This can be accomplished by placing the reaction system in a column oven.

Supelco offers a column water jacket that provides an inexpensive way of controlling temperature. The column and/or reaction system is placed in the water jacket and water from a circulating water bath travels through the jacket to maintain the desired temperature.

The effect of temperature on the reaction of aminoglycoside antibiotics is shown in Figure F. Response decreases at temperatures above 40°C due to rapid degradation of the reaction product.

Chromatographing the native compound allows one to fully exploit differences between analytes to maximize selectivity. Compounds that yield multiple products can still be quantitated as a single peak. Photochemical derivatization eliminates sample dilution and the problems associated with reagent delivery and mixing. Many classical wet chemical reactions can be adapted to postcolumn derivatization.

Ordering Information:

Postcolumn Reactor Products

Tee, Valco, 1/16"	
0.75mm bore	58283
0.25mm bore	58626
Single bead string reactor	
30cm x 0.5mm ID Teflon, filled with 250µm glass beads	
Acid washed beads	59204
Acid washed/silanized beads	59205
Mixing column hardware kit	58319
5cm x 4.6mm ID column blank, 2 fittings, 2 frits, 2" of 1/16" tubing	
TFE Teflon tubing, 10 feet	
1/16" x 0.3mm ID	58702
1/16" x 0.5mm ID	58701
1/16" x 0.8mm ID	58700
Glass beads, 25g	
Acid washed, 75µm	59200
Acid washed, 250µm	59202
Acid washed/silanized, 75µm	59201
Acid washed/silanized, 250µm	59203
Internal union, Valco, 1/16"	
(for terminating single bead string reactors)	
0.75mm bore	22997
0.25mm bore	58627
Glass wool, silane treated	20411
(for terminating single bead string reactors)	
Delay Tubes	
(knitted TFE Teflon tubing)	
10' x 0.5mm ID	59206
10' x 0.8mm ID	59207
Pulse damper, LO-Pulse®	58455
Column water jacket	58450
SSI Model 505 Column Oven	
100 VAC	59217
110 VAC	59215
220 VAC	59216

Low Volume Static Mixer

Increases reaction efficiency in postcolumn derivitization and improves microbore gradient accuracy. Mixer cartridges interchangeable. We recommend the 250µL cartridge for large peak volumes, and the 50 or 150µL sizes for smaller volumes.

Mixer cartridge	
50µL	57545
150µL	57546
250µL	57547
In-line housing	57548
Binary input housing	57549

Photochemical Reactor Enhancement Detection (PHRED) System



913-0355

Photochemical Reaction Products

PHRED photochemical reactor, 110V	57400
Replacement lamp bulb	57401
Knitted reactor coils	
5m x 0.25mm ID (0.25mL)	57402
10m x 0.25mm ID (0.5mL)	57403
15m x 0.25mm ID (0.75mL)	57404
20m x 0.25mm ID (1.0mL)	57410
5m x 0.50mm ID (1.0mL)	57405
10m x 0.50mm ID (2.0mL)	57406
15m x 0.50mm ID (3.0mL)	57407
20m x 0.50mm ID (4.0mL)	57411
Reflective support plate (stainless steel)	57408
Anti-UV safety glasses	57409

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Teflon – E.I. du Pont de Nemours & Co., Inc.
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