cLC™ System: An Automated Solution for Normal- and Reverse-Phase Prep HPLC with Analytical Purification Determination

Application Note 204

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Introduction

Reverse-Phase (RP) chromatography is the method of choice for purification of compounds. There are many manufacturers of semi- and preparative RP columns which range in IDs from 22 mm to 25 cm and lengths well over 250 mm (sample load range 25 mg–2.5 g). The flow rate range for these column sizes is 25–200 mL/min. Depending on solubility, sample loading is in the mg to low gram range. Because sample loading and drying down of the fractions collected can be a significant issue, many researchers employ Normal-Phase (NP) chromatography for some of their compounds.

NP columns are available in similar sizes and flow rate ranges as the RP columns. An additional option for NP chromatography is disposable columns; ID range of 25–75 mm and lengths from 7.5–30 cm (sample load range 80 mg–15 g based on ΔCV). These columns run at lower pressures (based on packing material). Although disposable columns are usually one-time use columns, they offer a much greater degree of sample loading, dry down is minimal (as with all NP HPLC), and they can be stored if further evaluation is required. Being able to accommodate both RP and NP chromatography usually requires two systems.

The cLC™ (complete LC) System presented in this application is a single-chromatography system capable of automating the purification of compounds using both RP columns and NP disposable and non-disposable columns. The cLC System also has the ability to accommodate various modes of detection, including UV, MS, and ELSD. The system is entirely automated through the software, excluding the user-defined manual loading of the disposable NP columns. Analytical components can also be integrated into the cLC System, enabling automated on-line evaluation of the collected fractions.

Materials & Methods

Instruments and Accessories

Pumping System: Prep—Gilson 333/334 Pump, equipped with four-position solvent selection valve (flow rates up to 200 mL/min.); Analytical—Gilson 32X Series Pump (flow rate to 30 mL/min.)

Gilson 215 Liquid Handler, equipped with: 175-mm arm, 10-mL dilutor syringe, 819 Injection Module (2), 7010 Rheodyne Valves, "make-before-break" stator and large-bore rotor seal, 10-mL loop (preparative), 20-µL loop (analytical), 5 racks (accommodating 13 x 150 mm and 18 x 150 mm tubes), and low-mount high-flow preparative collection valve

Columns: RP—CombiPrep™ C-18 (22 x 50 mm, 5 µ, 120A); NP—Betasil® Silica (20 x 150 mm, 5 µ, 100A), Biotage, Inc. Flash™ 40+M (40 x 150 mm, 100 g silica weight); Analytical—Supelco (4.6 x 150 mm, 5 µ, 100A)

Detector: Prep—Gilson 155 UV/VIS Dual-wavelength Detector, equipped with preparative flow cell (0.05-mm path length); Analytical—155 UV/VIS Dual-wavelength Detector, equipped with analytical flow cell (5.0-mm path length)

Gilson VALVEMATE® Valve Actuator(2), equipped with 7060 Rheodyne Valves (6-position column selection, large-bore stator and rotor seal)

Gilson UniPoint™ LC System Software, version 3.3

Gilson 506C System Interface Module

Intel® Pentium® 4 Processor (>2 GHz, 512 MB RAM, 80 GB hard drive)

Photo 1: Gilson's cLC™ System

The 215 Liquid Handler with low-mount, high-flow fraction collection valve offers a separate flow path from the injected sample, which eliminates any cross contamination between the sample and collected fractions. A planar arrangement is accommodated within 60" of bench space.

Control Methods

Control Method [change.gct]

Time	Device(s)	Command
0.02	Column Selector Inlet	Set Valve Position 5
0.04	Column Selector Outlet	Set Valve Position 5
0.06	Data Channels	Start Chromatogram Channels
0.10	Pump 1/Pump 2	0(ml/min): 50% Pump 1, 50% Pump 2
0.50	Pump 1/Pump 2	FL(ml/min): 50% Pump 1, 50% Pump 2
7.00	Data Channels	Stop Chromatogram Channels
7.00	Pump 1/Pump 2	FL(ml/min): 50% Pump 1, 50% Pump 2
8.00	Pump 1/Pump 2	0(ml/min): 50% Pump 1, 50% Pump 2

Table 1: Solvent Change Method for Switching Systems and Equilibration of the Instrument

The method shown in Table 1 ("CHANGE") enables the common solvent (isopropanol) to flush through the system, allowing for the conversion between NP and RP columns and vice versa. This method can also be run a second time under chromatographic conditions to stabilize the system prior to equilibrating the column with the mobile phase.

Control Method [equil.gct]

Time	Device(s)	Command
0.01	Column Selector Inlet	Set Valve Position Variable SELECT_COLUMN
0.03	Column Selector Outlet	Set Valve Position Variable SELECT_COLUMN
0.05	Pump 1/Pump 2	0(ml/min): Pump 1, INIT_ORG% Pump 2
0.07	Detector 16	Set Mode Dual
0.09	Detector 16	Set Dual Wavelength 1 254
0.11	Detector 16	Set Dual Wavelength 2 280
0.13	Detector 16	Set Dual Sensitivity 1 1.0
0.15	Detector 16	Set Dual Sensitivity 2 1.0
0.17	Detector 16	Autozero Channels
2.00	Data Channels	Start Chromatogram Channels
3.00	Pump 1/Pump 2	FL(ml/min): Pump 1, INIT_ORG% Pump 2
9.00	Data Channels	Stop Chromatogram Channels
10.00	Pump 1/Pump 2	FL(ml/min): Pump 1, INIT_ORG% Pump 2

Table 2: Solvent Change Method for Switching Systems and Equilibration of the Instrument

The "EQUIL" method (shown in Table 2) will allow the column to equilibrate with initial mobile phase conditions prior to the injection of the sample. This method should also run between similar columns (e.g., multiple RP or NP columns).

Control Method [np_clc.gct]

Time	Device(s)	Command
0.01	Pump 1/Pump 2	0(ml/min): Pump 1, INIT_ORG% Pump 2
0.03	Injector	Set Injection Valve Position Load
0.05	WAITING FOR INJECTION	Wait
0.10	Pump 1/Pump 2	0(ml/min): Pump 1, INIT_ORG% Pump 2
0.12	Detector 16	Autozero Channels
0.14	Data Channels	Start Chromatogram Channels
0.16	Fraction Collector	Set Collection and Travel Depths 3, 3
0.18	Fraction Collector	Set Peak Level PK_LVL
0.20	Fraction Collector	Set Fraction by Volume Inside a Peak FC_VOL
0.22	Fraction Collector	Set Fraction Site FC_SITE
0.24	Fraction Collector	Start Collection
0.70	Pump 1/Pump 2	FL(ml/min): Pump 1, INT_ORG% Pump 2
7.00	Pump 1/Pump 2	FL(ml/min): Pump 1, END_ORG% Pump 2
9.00	Pump 1/Pump 2	FL(ml/min): Pump 1, END_ORG% Pump 2
39.00	Fraction Collector	Stop Collection
39.50	Data Channels	Stop Chromatogram Channels
40.00	Pump 1/Pump 2	FL(ml/min): Pump 1, INT_ORG% Pump 2

Table 3: NP Chromatography for the Biotage FLASH 40+M Columns

The Biotage FLASH 40+M columns are loaded manually, either off line or on the system prior to use. Manual loading of the sample onto the column is one of several loading techniques suggested by Biotage. Isocratic parameters are usually employed for NP chromatography. However, gradient profiles are available in the software. There are three ways to load the Biotage FLASH columns:

- Load the sample dissolved in a given amount of solvent (usually less than 20 mL)
- Load the sample in a samplet (similar to a guard cartridge) and drop it in the top of the cartridge
- Load the sample dry by packing the top of the cartridge

Control Method [rp_clc.gct]

Time	Device(s)	Command
0.01	Pump 1/Pump 2	FL(ml/min): 100% Pump 1, 0% Pump 2
0.10	Partial loop fill for 215 as FC	<start> INJECT_VOLUME, SAMPLE
0.36	System Controller	Synchronize
0.38	Detector 16	Autozero Channels
0.40	Data Channels	Start Chromatogram Channels
0.50	Pump 1/Pump 2	FL(ml/min): 100% Pump 1, 0% Pump 2
1.35	System Controller	Synchronize
1.37	Fraction Collector	Set Collection and Travel Depths 3, 3
1.39	Fraction Collector	Set Peak Level PK_LVL
1.41	Fraction Collector	Set Fraction by Volume Inside a Peak FC_VOL
1.43	Fraction Collector	Set Fraction Site FC_SITE
1.45	Fraction Collector	Start Collection
7.00	Pump 1/Pump 2	FL(ml/min): 5% Pump 1, 95% Pump 2
9.00	Pump 1/Pump 2	FL(ml/min): 5% Pump 1, 95% Pump 2
10.50	Pump 1/Pump 2	FL(ml/min): 100% Pump 1, 0% Pump 2
11.30	Fraction Collector	Stop Collection
11.50	Data Channels	Stop Chromatogram Channels
12.00	Pump 1/Pump 2	FL(ml/min): 100% Pump 1, 0% Pump 2

Table 4: RP Chromatography

As shown in Table 4, a gradient of increasing organic is initiated to minimize the run time and sharpen the peaks. Variables are used so that various gradients and parameters for fraction collection could be explored and optimized for individual samples between runs.

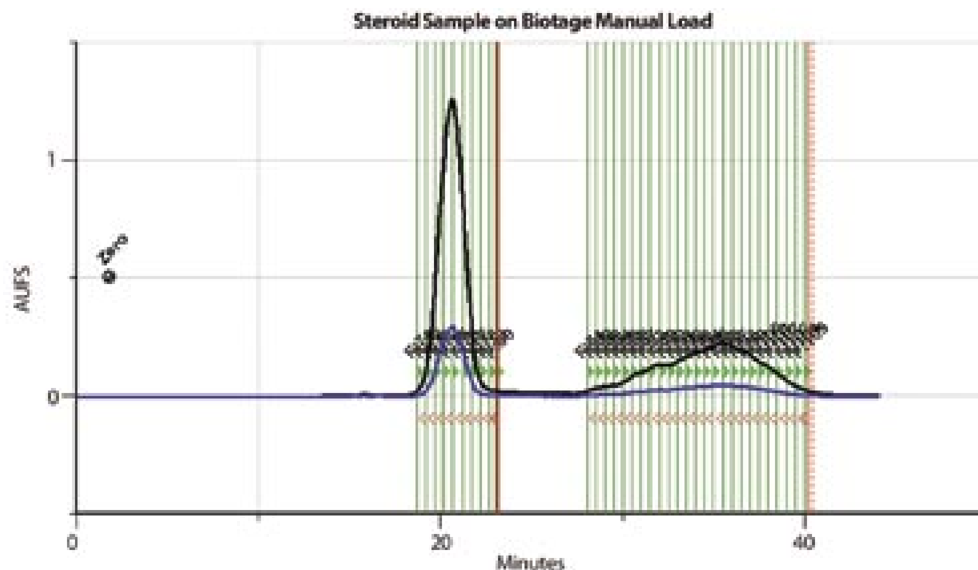
Operations [clc_sys.gop]

Description	Control Method	Analysis Method	FL	Column	Volume	Sample	PK_LVL	FC_VOL	FC_SITE	INIT_ORG	END_ORG
Start System	CLC\EQUIL.GCT		40	5						98	
Equilibrate NP System	CLC\EQUIL.GCT		40	6						98	
Start System	CLC\EQUIL_NP.GCT		40	6						98	
Estriol + Estrone	CLC\NP_INJFC.GCT	NP_CLC.GAN	40		2000	S:1	100	15	F:1	98	80
Change System	CLC\CHANGE.GCT		50								
Equilibrate RP System	CLC\EQUIL.GCT		30	1						0	
RP_Mixture	CLC\RP_CLC.GCT	RP_CLC.GAN	30		4000	S:2	100	20	F:		
Equilibrate RP System	CLC\EQUIL.GCT		30	2						0	
RP_Mixture	CLC\RP_CLC.GCT	RP_CLC.GAN	30		4000	S:3	100	15	F:		
Equilibrate RP System	CLC\EQUIL.GCT		30	3						0	
RP_Mixture	CLC/RP_CLC.GCT	RP_CLC.GAN	30		4000	S:4	50	20	F:		
Equilibrate RP System	CLC/EQUIL.GCT		30	4						0	
RP_Mixture	CLC\RP_CLC.GCT	RP_CLC.GAN	30		4000	S:5	50	15	F:		
Wash RP System	CLC\SHUTDOWN.GCT		25								
Common Solvent Rest	CLC\CHANGE.GCT		40								
Change to NP	CLC\EQUIL.GCT		35	5						100	
Change to NP	CLC\EQUIL.GCT		35	5						100	
Change to NP Equilibrate Biotage	CLC\EQUIL.GCT		30	6						100	
Change to NP Equilibrate Biotage	CLC\EQUIL.GCT		30	6						100	
Steroid Sample on Biotage Manual Load	CLC\NP_CLC.GCT	NP_CLC.GAN	30				100	20	F:	80	80

Table 5: Operations List for the cLC System

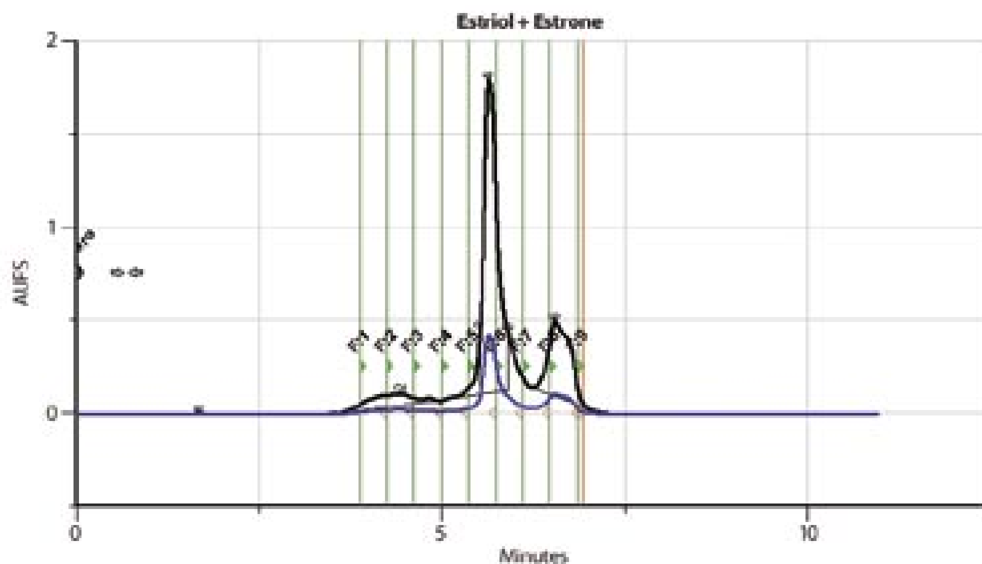
Table 5 shows the sequence of steps involved in the automated operations of the cLC System. Without operator intervention, the system will chromatograph a series of samples under NP conditions, then stabilize and equilibrate to run samples under RP conditions. The system can then switch back to NP and run additional Biotage FLASH columns that were prepared while RP samples were running.

Performance Data



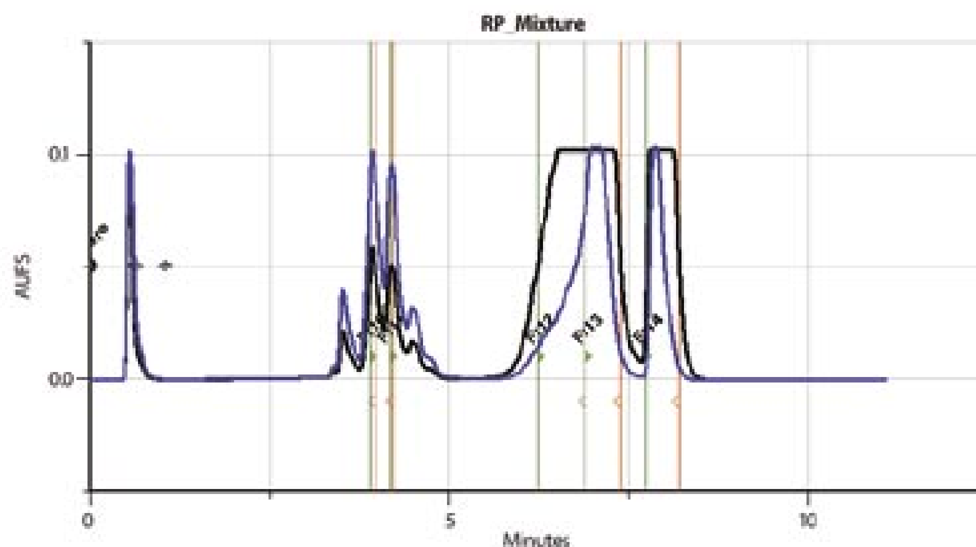
Graph 1: Biotage FLASH™ 40+M Column

The NP chromatogram (in Graph 1) was produced from the Biotage FLASH 40+M column. The column was manually loaded with 15 mL of solution (THF/dioxane) in which 445 mg of sample was dissolved (estrone: 318 mg and estriol: 127 mg), at a flow rate of 30 mL/min., hexane/IPA (90:10).



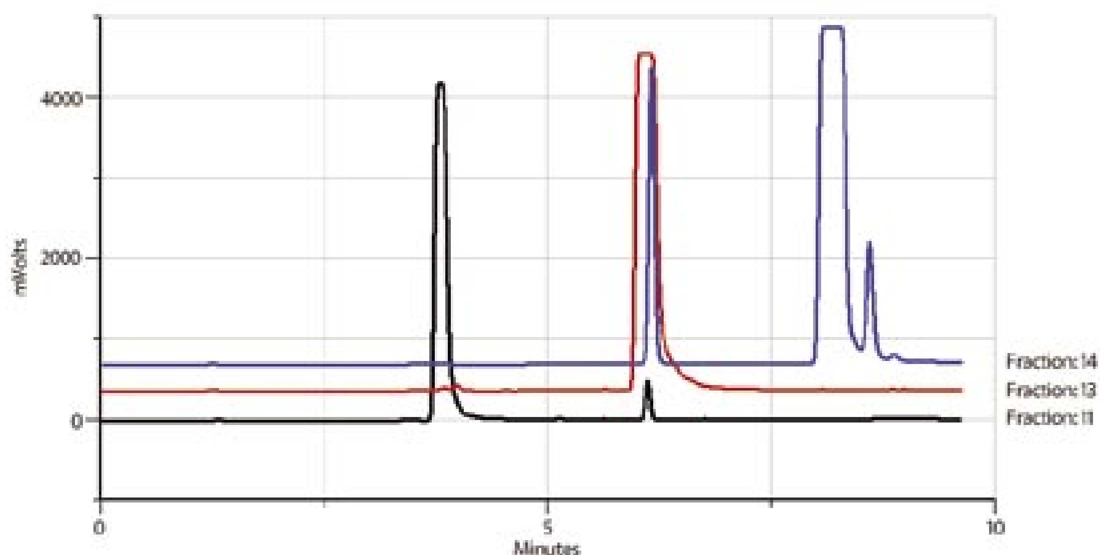
Graph 2: Betasil® Silica Column

The NP chromatogram (in graph 2) was achieved with a Betasil silica column (20 x 150 mm, 5 µ, 100A). The sample (156 mg) was estrone (96 mg) and estriol (60 mg) in a 4-mL injection on column. An isocratic run of hexane/IPA (98:2) chromatographed the sample in less than 10 minutes.



Graph 3: RP-CombiPrep™ Pro C18 Column

The RP chromatogram (in graph 3) represents the chromatography established for a mixture of three components: caffeine, 102 mg; p-hydroxy benzoic acid ethyl ester, 68 mg; biphenyl, 69 mg (total on column injection of 239 mg in a 4-mL injection volume). A gradient of 5–95% water/ACN was run over 7 minutes. Fractions collected were injected under analytical conditions to determine the components of the fractions collected.



Graph 4: Injection of Fractions Collected from RP Chromatography

Fractions collected from the RP-CombiPrep column were injected (without dry down) on line onto an analytical system. The samples were run on a Supelco analytical column (4.6 x 150 mm, 5 µ, 100A), at 1.5 mL/min., 5–95% water/acetonitrile over 7 minutes. 40 µL of each fraction was injected onto the column. The overlay shows the separation of the three components that were injected onto the semi-preparative column from the fractions collected (see Graph 4).

Summary

The cLC™ system is capable of handling up to 5 columns (NP and RP) with one bypass for quick changeover between the two types of chromatography. The system can accommodate disposable NP chromatography columns (sample load <15 g, based on ΔCV) (e.g., Biotage FLASH 24+M to 75M) in an HPLC environment which will accommodate other modes of detection: MS, ELSD (which is not available with off-line split stream instruments).

The cLC system is totally automated and accommodates the wide range of solvents used through solvent selection valves without operator intervention. Although most NP chromatography is run under isocratic conditions, the cLC system offers the advantage of improving NP Flash chromatography via gradients. NP gradients are being run at the present time in several pharmaceutical labs on the cLC system employing the Biotage FLASH columns.

The addition of analytical components allows for the checking of the fractions collected on line, prior to dry down. Additional solvent and column selection valves could be used on the analytical system to allow not only the checking of the RP fractions, but the NP fractions as well.

Conclusion

Gilson's cLC system offers researchers the capabilities of both reverse-phase and normal-phase chromatography in one system for about the same investment made for one preparative HPLC system. Its footprint is only slightly larger than a preparative system, and many of the components can be stacked, minimizing the bench space used. The ability to switch the solvent and column selector valves to any position, without having to move sequentially (clockwise or counterclockwise), substantially reduces solvent incompatibilities and avoids column contamination problems.

The addition of an analytical HPLC system allows for on-line analysis of collected fractions from the preparative run.

Since the system is made up of true HPLC components, it is capable of running all modes of chromatography: low-, medium-, and high-pressure columns depending on the application. With this in mind, the cLC system represents a merger of two systems into one HPLC with off-line, low-pressure chromatography that includes numerous features and benefits not available in the lower-pressure chromatography instruments.

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