

## High-Speed LC/MS Analysis with MercuryMS™ Cartridges for High-Throughput Drug Discovery

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### Introduction

The ever increasing demand for high-throughput screening (HTS) of drug candidates during the early stages of drug discovery has generated an acute need for rapid methods of analysis. Moreover, preclinical trials, which generate large volumes of samples comprising drug/metabolite combinations, also require fast analysis turnaround for obtaining pertinent pharmacokinetic information. Developing ultra-fast and effective methods for HTS of potential drugs has become a constant challenge for analysts. The combination of HPLC separation with MS/MS detection (LC/MS/MS) has become the most powerful and preferred technique for undertaking the high-throughput analysis of combinatorial libraries and preclinical pharmacokinetic studies. Because of its sensitivity, selectivity, and robustness, LC/MS/MS has provided the much needed speed for HTS, but also contributed to rapid advances in instrumental design in the area of separation sciences. Besides miniaturization and improved separation efficiency, speed has become the driving force for HPLC separations during drug discovery. We address this need for high speed by presenting in this technical note, data on the performance of the MercuryMS™ Cartridge system in high-speed LC/MS/MS gradient separations for a wide range of pharmaceutical compounds or metabolites in mixtures, with cycle times of 1.2 or 2 minutes.

### Experimental Conditions

#### Instrumentation and Configurations

**HPLC System:** HP 1100 series (www.agilent.com)  
**Pump:** G1312A (Binary Pump) without in-line mixer  
**UV Detector:** G1315A DAD with semi-micro flow cell - 6mm path (5µL)  
**MS Detector:** API 3000 LC/MS/MS System (www.appliedbiosystems.com), ESI+ (Turbolonspray), MRM  
**Injector(Autosampler):** G1329A ALS with Needle Seat Capillary (0.12mm id -red)

**Connecting tubing:** Pump to Injector 55cm x 0.125mm (red PEEK tubing); Needle seat/capillary 0.12mm id, 1.2µL (red PEEK tubing); Injector to Column 28cm x 0.12mm (red PEEK tubing); Column to DAD 28 cm x 0.12mm (red PEEK Tubing); Column to MS detector 53cm x 0.12mm (red PEEK tubing).

#### HPLC System Configurations before Optimization

**Pump:** G1312A (Bin Pump) with in-line Mixer  
**Detector:** G1315A DAD with standard flow cell -10mm path (13µL)  
**Injector(Autosampler):** G1329A ALS with Needle Seat Capillary (0.17mm id -green)

**Connecting tubing:** Pump to Injector 55cm x 0.17mm (green PEEK tubing); Needle seat 0.17mm id 2.3µL (green PEEK tubing); Injector to Column 28cm x 0.17mm (yellow PEEK tubing); Column to DAD 28 cm x 0.12mm (red PEEK Tubing).

#### LC Conditions

**Flow rate:** 0.6mL/min or 2.5mL/min (indicated on each chromatogram)  
**Mobile Phase:** A: 0.1% Formic Acid in Water, B: 0.1% Formic Acid in Acetonitrile  
**Injection:** 1.0 or 2.0 µL (as indicated on each chromatogram)  
**UV Detection:** 254nm  
**Temperature:** Ambient

#### MS Conditions

Turbolonspray heater gas flow: 6500cc/min  
 Turbolonspray heater temperature: 425 - 450°C

#### Column or Cartridge

Phenomenex® Luna® C18(2) 3µm	20 x 2.0mm (cartridge and mini-column) and 20 x 4.0mm (cartridge)
Phenomenex® Synergi™ Fusion-RP 2µm	10 x 2.0 (cartridge)

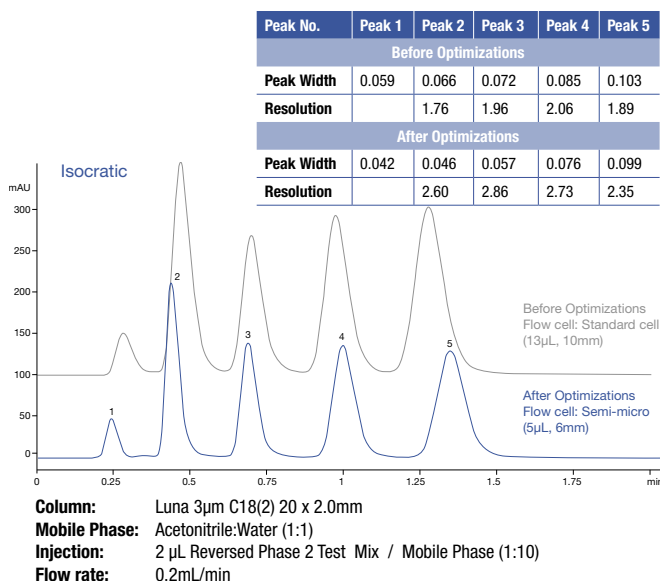
#### Recommended Injector Program (from Agilent™ technical literature)

Steps	Command	Comment
1	DRAW	Draw volume of sample (injection volume) from vial
2	INJECT	Introduce sample onto column
3	WAIT 0.06min (calculated wait time)	Flush sample loop after injection (wait time = 6 x (injection volume + 5µL)/flow rate)
4	VALVE bypass	Direct flow from pump to column bypassing injection valve to exclude delay volume (~300µL from auto-injector path)
5	WAIT 1.5min	The period of VALVE bypass time (Wait time = Runtime - 1min)
6	VALVE mainpass	Switch VALVE from bypass to injection position (path)

### Discussion of Results

Optimization of the HPLC system for high-throughput analysis is critical in order to minimize system delay volume and fully exploit the capabilities of these miniature, high performance cartridges or mini-columns. The consequences of performing chromatographic analysis using a standard system configuration as compared to an optimized system are broad peaks and decreased resolution (i.e. reduced peak capacity for cartridges and mini-columns) (**Figure 1**). Furthermore, by using an injector program the gradient delay time can be further decreased which is very useful at low flow rates (**Figure 2**).

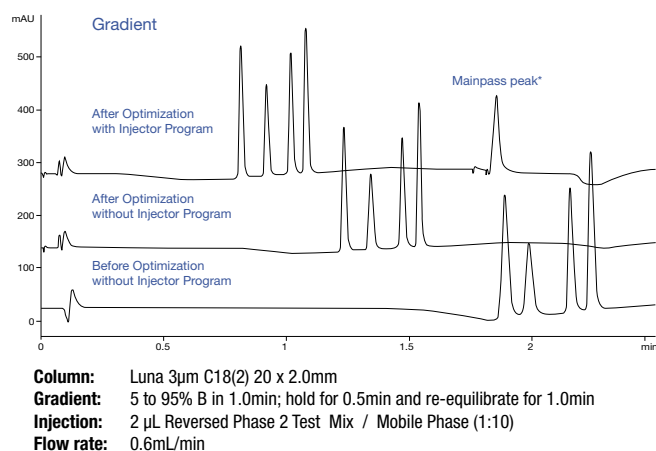
Figure 1. Effect of HPLC System Configuration (1)\*\*



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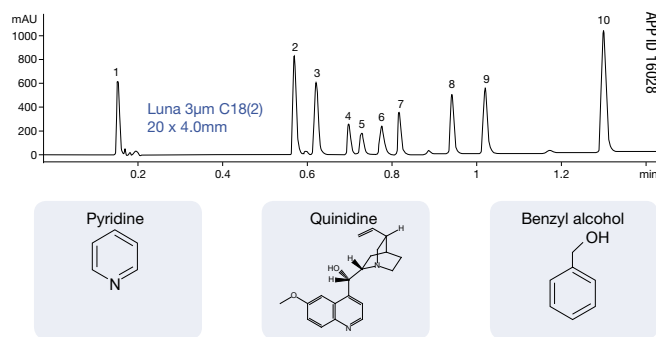
Phenomenex products are available worldwide. For the distributor in your country, contact Phenomenex USA, International Department by telephone, fax or e-mail: international@phenomenex.com.

**Figure 2. Effect of HPLC System Configuration (2)\*\***



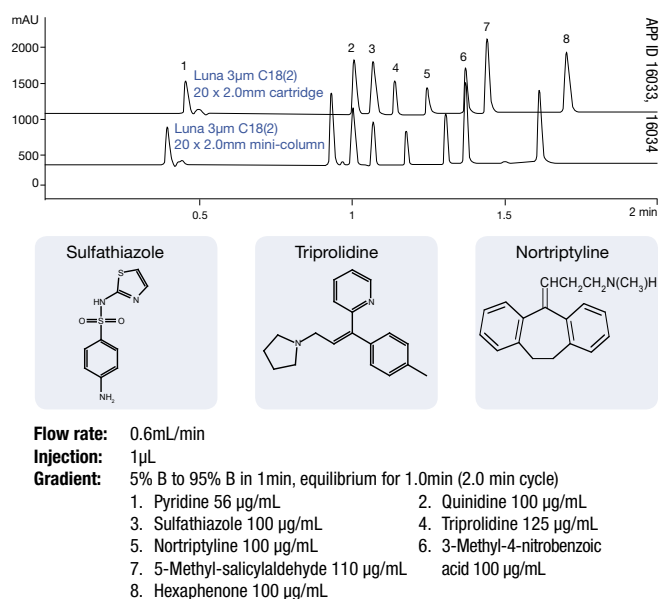
In order to study the performance of MercuryMS™ cartridges, mixtures of probes with divergent chemistries and polarities (acidic, neutral, and basic compounds) were analyzed by HPLC with UV detection (**Figure 3:** in 1.2 min cycle; **Figure 4** in 2.0 min cycle). The performance of Luna 3µm C18(2) in both MercuryMS™ cartridge and mini-column format was found to be comparable. The results show that Luna 3µm C18(2) MercuryMS™ cartridges and mini-columns exhibit excellent chromatographic performance with respect to peak shape, resolution and peak capacity.

**Figure 3. Acid, Neutral, and Basic Comp. (LC-UV) - (1)**



- |                                       |   |
|---------------------------------------|---|
| 1. Pyridine 56 µg/mL                  | 2. Quinidine 100 µg/mL                    |
| 3. Sulfathiazole 100 µg/mL            | 4. Tripolidine 125 µg/mL                  |
| 5. Benzyl alcohol 1.2 mg/mL           | 6. Phenol 0.5mg/mL                        |
| 7. Nortriptyline 100 µg/mL            | 8. 3-Methyl-4-nitrobenzoic acid 100 µg/mL |
| 9. 5-Methyl-salicylaldehyde 110 µg/mL |   |
| 10. Hexaphenone 100 µg/mL             |   |

**Figure 4. Acid, Neutral, and Basic Probes (LC-UV) - (2)**



Due to the high specificity of MS/MS or even single MS detection, resolution is no longer a major concern for high speed analysis. Ultrafast analyses can be achieved without compromising separation efficiency and selectivity. Therefore the running cycles can be further shortened to 1min, or even less, by splitting high flow rates to MS, while using ESI as the ionization source.

### Sample Preparation of Drug Compounds and Metabolites for High-Throughput Analysis

#### Benzodiazepines and Metabolites

**Sample:** Spiked different concentration levels of benzodiazepines, metabolites and 50ng/mL Clonazepam as IS into 200µL urine or human plasma; diluted to 1mL with water

**SPE:** Strata-X 30mg/mL (part number: 8B-S100-TAK)

**Conditioning:** 1mL methanol, followed by 1mL water

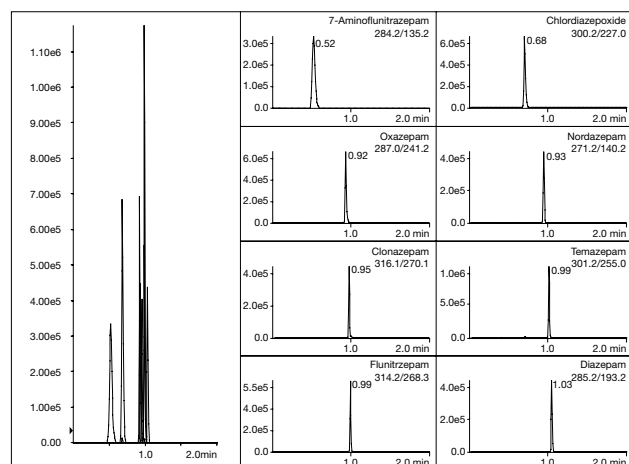
**Loading:** Load above spiked sample onto SPE cartridge

**Washing:** 1mL water, followed by 1mL 20% Methanol in water

**Eluting:** 1mL methanol; eluate evaporated to dryness. Reconstitute with 200µL 15% acetonitrile in water

\*\*For ordering information about HPLC Column Check Standards, contact your Phenomenex Technical Consultant.

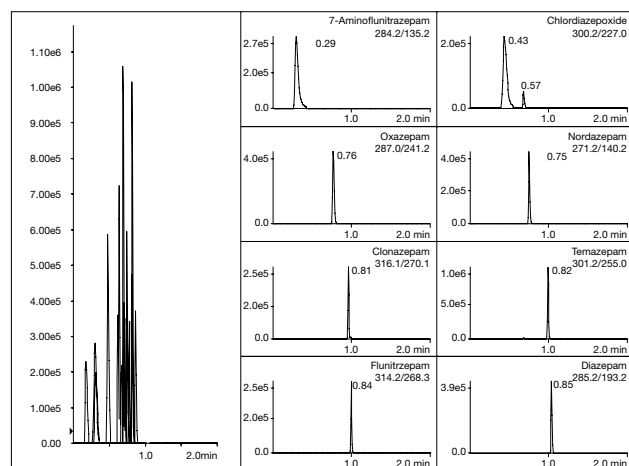
**Figure 5. Benzodiazepines in Plasma (LC/MS/MS - 1)**



APP ID 16040

**Column:** Luna 3µm C18(2) 20 x 2.0mm MercuryMS™ cartridge  
**Gradient:** 15:85 to 95:5% B in 1.0 min, re-equilibration for 1.0min (2.0min cycle)  
**Injection:** 10µL of 100ng/mL Benzodiazepines from human plasma after SPE clean-up  
**Flow rate:** 0.6mL/min

**Figure 6. Benzodiazepines in Urine (LC/MS/MS - 2)**



APP ID 16042

**Column:** Synergi 2µm Fusion-RP 10 x 2.0mm MercuryMS™ cartridge  
**Gradient:** 15:85 to 95:5% B in 1.0 min, re-equilibration for 1.0min (2.0min cycle)  
**Injection:** 10µL of 100ng/mL Benzodiazepines in urine after SPE clean-up  
**Flow rate:** 0.6mL/min

To demonstrate the feasibility of fast LC/MS/MS quantitative analyses of physiological samples in *in vitro* drug metabolism and drug toxicology studies, benzodiazepines, tricyclic antidepressants<sup>†</sup> and their metabolites were spiked into biological matrices of human origin for quantitative evaluation.

<sup>†</sup>Tricyclic antidepressant data available upon request.

Absolute recoveries were determined by comparing peak areas of same level standards with spiked drug or metabolite extracts. The absolute recoveries are higher than 55% for all of the spiked drugs and their metabolites at the 100ng/mL level (**Table 1**). Linearity was studied in the concentration ranges: 5-500 ng/mL for benzodiazepines (with IS - clonazepam at 50ng/mL). Results demonstrate good linearity in all cases with linear regression values of  $R^2 > 0.995$  (**Table 1**). Precision was studied by interassay or intraassay (benzodiazepines) RSD at 6 replicate assays. Results show that RSD are less than 15% at the 100ng/mL level in all cases (**Table 1**).

**Table 1. Quantification of Benzodiazepines from Plasma or Urine by LC/MS/MS High-Throughput Analysis**

Compounds	Chlordiazepoxide	Diazepam	Oxazepam	Temazepam
Structure				
Metabolites	Nordiazepam Oxazepam	Nordiazepam Temazepam Oxazepam		
MW	299.76	284.74	286.72	300.74
MRM	300.3/227.0	258.2/193.2	287.0/241.2	301.2/255.0
$R^2$ *1	<b>0.9994</b>	<b>0.9990</b>	<b>0.9990</b>	<b>0.9996</b>
RSD% *2	3.28 ~ 11.74	5.43 ~ 13.62	4.85 ~ 5.15	5.06 ~ 9.63
% Recovery *2 (Absolute)	54.75 ~ 66.54	59.28 ~ 77.87	57.22 ~ 68.63	59.57 ~ 86.80

**Note:**

\*1. Concentration range: 5-500ng/mL by spiking 6pts into human plasma, one set.

\*2. n=6 at 100ng/mL in urine or plasma, by interassay

Compounds	7-Amino - flunitrazepam	Flunitrazepam	Nordazepam	Clonazepam
Structure				
Metabolites		7-Amino - flunitrazepam		
MW	283.30	313.29	270.71	315.72
MRM	284.3/135.2	314.2/268.3	271.2/140.2	316.1/270.1
$R^2$ *1	<b>0.998</b>	<b>0.997</b>	<b>0.998</b>	<b>IS</b>
RSD% *2	5.41 ~ 10.00	7.74 ~ 11.74	5.33 ~ 8.67	7.93 ~ 11.08
% Recovery *2 (Absolute)	55.60 ~ 69.02	64.34 ~ 81.16	65.25 ~ 74.16	57.87 ~ 68.81

**Note:**

\*1. Concentration range: 5-500ng/mL by spiking 6pts into human plasma, one set.

\*2. n=6 at 100ng/mL in urine or plasma, by interassay

## Conclusions

The performance of MercuryMS™ Cartridges for the analysis of complex mixtures of acidic, basic and neutral compounds was successfully demonstrated by HPLC(UV) with short cycle times. The chromatograms with UV detection show good resolving power, and good peak shape. MercuryMS™ Cartridges provide the performance of analytical size columns while shortening significantly the cycle time.

HPLC system configuration is critical to achieving maximum performance in high-throughput analysis – binary systems without in-line mixers, and using injector programs, are optimal. The use of MercuryMS™ cartridges for the quantitative LC/MS/MS analysis of common pharmaceutical compounds (benzodiazepines and their metabolites) with fast gradients (2.0 min cycle or less) was demonstrated. Luna C18(2) and the Synergi line of MercuryMS™ cartridges provide the desired features of speed and selectivity for high-throughput analysis in the drug discovery and the related DMPK areas.

## Ordering Information

Synergi 2µm in MercuryMS Column Format (mm)				
Phase	20 x 2.0	20 x 4.0	50 x 2.0	50 x 4.6
Max-RP	00M-4372-B0	00M-4372-D0	00B-4372-B0	00B-4372-E0
Hydro-RP	00M-4387-B0	00M-4387-D0	00B-4387-B0	00B-4387-E0
Polar-RP®	00M-4371-B0	00M-4371-D0	00B-4371-B0	00B-4371-E0
Fusion-RP	00M-4423-B0	00M-4423-D0	00B-4423-B0	00B-4423-E0

Synergi 2µm in MercuryMS Cartridge Format (mm)				
Phase	10 x 2.0	10 x 4.0	20 x 2.0	20 x 4.0
Max-RP	00N-4372-B0-CE	00N-4372-D0-CE	00M-4372-B0-CE	00M-4372-D0-CE
Hydro-RP	00N-4387-B0-CE	00N-4387-D0-CE	00M-4387-B0-CE	00M-4387-D0-CE
Fusion-RP	00N-4423-B0-CE	00N-4423-D0-CE	00M-4423-B0-CE	00M-4423-D0-CE

3µm in MercuryMS Column Format (mm)				
Phase	20 x 2.0	20 x 4.0	30 x 2.0	50 x 2.0
Luna C18 (2)	00M-4251-B0	00M-4251-D0	00A-4251-B0	00B-4251-B0
Luna Phenyl Hexyl	00M-4256-B0	00M-4256-D0	00A-4256-B0	00B-4256-B0
Gemini C18	00M-4439-B0	00M-4439-D0	00A-4439-B0	00B-4439-B0
Gemini C6-Phenyl	00M-4443-B0	00M-4443-D0	00A-4443-B0	00B-4443-B0

3µm in MercuryMS Cartridge Format (mm)				
Phase	10 x 2.0	10 x 4.0	20 x 2.0	20 x 4.0
Luna C18 (2)	00N-4251-B0-CE	00N-4251-D0-CE	00M-4251-B0-CE	00M-4251-D0-CE
Luna Phenyl Hexyl	00N-4256-B0-CE	00N-4256-D0-CE	00M-4256-B0-CE	00M-4256-D0-CE
Gemini C18	00N-4439-B0-CE	00N-4439-D0-CE	00M-4439-B0-CE	00M-4439-D0-CE
Gemini C6-Phenyl	00N-4443-B0-CE	00N-4443-D0-CE	00M-4443-B0-CE	00M-4443-D0-CE



### Standard Cartridge Holder

Part No.	Description
CHO-5846-TN	10mm standard holder
CHO-5845-TN	20mm standard holder

### Direct-Connect Cartridge Holders

Part No.	Description
CHO-7187-TN	10mm direct-connect holder
CHO-7188-TN	20mm direct-connect holder

### strata™X SPE Sorbent

Part No.	Description
8B-S100-TAK-TN	strata-X 30mg/1mL Tubes (100/Box)
8B-S100-UBJ-TN	strata-X 60mg/3mL Tubes (50/Box)
8B-S100-FBJ-TN	strata-X 200mg/3mL Tubes (50/Box)
8B-S100-HBJ-TN	strata-X 500mg/3mL Tubes (50/Box)
8B-S100-ECH-TN	strata-X 100mg/6mL Tubes (30/Box)
8B-S100-FCH-TN	strata-X 200mg/6mL Tubes (30/Box)
8B-S100-HCH-TN	strata-X 500mg/6mL Tubes (30/Box)
8B-S100-HDG-TN	strata-X 500mg/12mL GigaTubes (20/Box)
8B-S100-JEG-TN	strata-X 1g/20mL GigaTubes (20/Box)
8E-S100-NGB-TN	strata-X 96-Well Plate 5mg/well (2/Box)
8E-S100-AGB-TN	strata-X 96-Well Plate 10mg/well (2/Box)
8E-S100-TBG-TN	strata-X 96-Well Plate 30mg/well (2/Box)
8E-S100-UGB-TN	strata-X 96-Well Plate 60mg/well (2/Box)
00M-S033-B0-CB-TN	strata-X 25µm On-Line Extraction Cartridge (each)
CHO-5845-TN	20mm Cartridge Holder (each)