# Method Development for Reversed Phase Chiral LC/MS/MS Analysis of Stereoisomeric Pharmaceutical Compounds with Polysaccharide-based Stationary Phases

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*Five different Lux® polysaccharide-based chiral stationary phases were explored in the reversed phase elution mode using mobile phases consisting of 0.1 % formic acid in acetonitrile or methanol to demonstrate the feasibility of LC/MS/MS analysis of a variety of acidic pharmaceutical racemates.*

## Introduction

Developing simple and straightforward reversed phase chiral LC separations coupled with highly sensitive MS detection is a challenging requirement for conducting drug metabolism and pharmacokinetic studies of stereoisomers. Polysaccharide derivatives are the most widely used chiral stationary phases (CSP) due to their wide chiral recognition and high loading capacity. As normal phase is favorable for their principal mechanism of chiral recognition – hydrogen bonding interaction – the majority of chiral separations with polysaccharide phases are performed in normal phase using hexane and alcohol modifiers as mobile phase components. However, these mobile phases are highly flammable and are not compatible with atmospheric pressure ionization (API) MS ion sources. The current research describes the effectiveness of an acidic mobile phase for the separation and detection of using acidic stereoisomers by ESI or APCI LC/MS/MS and for method development of these applications.

## Experimental

Analytes: 15 acidic compounds of pharmaceutical interest were analyzed. The structures are shown in **Figure 2** and the MS ionization and mass reaction monitoring (MRM) transitions monitored are listed in **Table 1.**

**Columns:** Lux 3 µm Cellulose-1, 150 x 2.0 mm
Lux 3 µm Cellulose-2, 150 x 2.0 mm
Lux 3 µm Amylose-2, 150 x 2.0 mm
Lux 5 µm Cellulose-1, 250 x 4.6 mm
Lux 5 µm Cellulose-4, 250 x 4.6 mm
Lux 5 µm Cellulose-3, 250 x 4.6 mm
Lux 5 µm Amylose-2, 250 x 4.6 mm
Kinetex® 2.6 µm C18, 50 x 2.1 mm (used for achiral analysis)

**Flow Rate:** 0.2 mL/min (3 µm, 150 x 2.0 mm) or
1.0 mL/min (5 µm, 250 x 4.6 mm) – flow split to 0.25 mL/min into MS/MS

**Temperature:** 25 °C

**Detection:** UV @ 220 nm

**Injection Volume:** 5 µL (150 x 2.0 mm) or 10-20 µL (250 x 4.6 mm)

**Mobile Phases:** 1. 0.1 % Formic acid in Acetonitrile or Methanol
2. 5 mM Ammonium bicarbonate in Acetonitrile or Methanol (achiral analysis)
3. 5 mM Ammonium formate in Acetonitrile or Methanol (achiral analysis)
4. 5 mM Ammonium acetate in Acetonitrile or Methanol (achiral analysis)

**Instrument:** HPLC System: Agilent® 1200 series equipped with binary pump and autosampler (Agilent, Palo Alto, CA)
MS Detector: AB SCIEX™ 4000 LC/MS/MS
Turbo V™ source with ESI or APCI probe
MS Detection: TurbolonSpray® – ESI or APCI in Positive or Negative Ion Mode; MRM

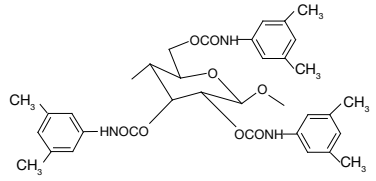
## Results and Discussion

*Chiral LC/MS/MS Experiments*

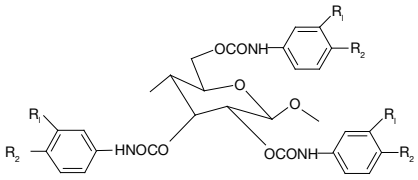
Five different polysaccharide-based chiral stationary phases (Lux Cellulose-1, Lux Cellulose-2, Lux Cellulose-3, Lux Cellulose-4, and Lux Amylose-2 **(Figure 1)** were explored in the reversed phase elution mode for the separation of a variety of acidic compounds of pharmaceutical interest in mobile phases consisting of 0.1% formic acid in acetonitrile or methanol and MS/MS detection.

**Figure 1**.
Structures of Polysaccharide-based Chiral Stationary (CSPs) Phases

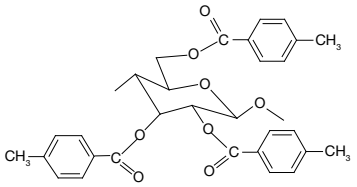
Lux Cellulose-1
Cellulose tris (3,5-dimethylphenylcarbamate)



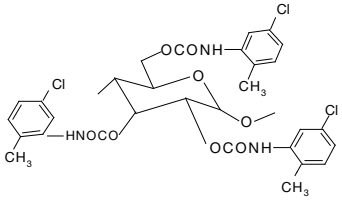
Lux Cellulose-2
Cellulose tris (3-chloro-4-methylphenylcarbamate)
with $R_1$=Cl and $R_2$=$CH_3$
or
Lux Cellulose-4
Cellulose tris (4-chloro-3-methylphenylcarbamate)
with $R_1$=$CH_3$ and $R_2$=Cl.



Lux Cellulose-3
Cellulose tris-(4-methylbenzoate)



Lux Amylose-2
Amylose tris (5-chloro-2-methylphenylcarbamate)

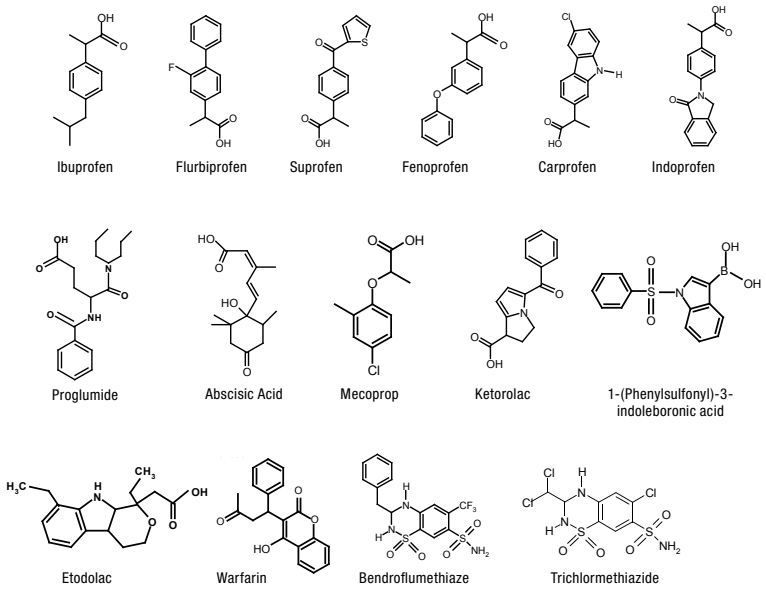


# APPLICATIONS

**Table 1.**
MRM Transitions and Concentrations of Acidic Racemates

| Compound | IS and MRM | Conc.* | Compounds | IS and MRM | Conc.* |
|---|---|---|---|---|---|
| Ibuprofen | ESI⁻205.1/160.1 | 100 | Abscisic acid | APCI⁻263.0/152.7 | 50 |
| Flurbiprofen | ESI⁻243.0/198.7 | 50 | Mecoprop | ESI⁻214.1/141.7 | 50 |
| Suprofen | ESI⁺261.1/111.0 | 50 | Ketorolac | ESI⁻254.0/209.8<br>ESI⁺256.2/105.0 | 50 |
| Fenoprofen | APCI⁻241.0/196.8 | 100 | Etodolac | ESI⁻286.1/242.0 | 50 |
| Carprofen | APCI⁻272.8/228.8 | 100 | Warfarin | ESI⁻307.1/160.9<br>ESI⁺309.2/163.1 | 50 |
| Indoprofen | ESI⁺282.1/236.1 | 50 | Bendroflumethiaze | ESI⁻420.1/77.9 | 50 |
| Proglumide | ESI⁻333.1/120.9 | 50 | Trichlormethiazide | ESI⁻377.8/241.6 | 50 |
| 1-(Phenylsulfonyl)-3-indoleboronic acid | ESI⁻300.0/209.8 | 100 | | | |
| | | | * Conc. (ng/mL) | | |

**Figure 2**.
Molecular Structures of Acidic Racemates




*Selection of Mobile Phase Additives*
As acidic analytes are present as anions in mobile phases of neutral pH their retention is not favorable on polysaccharide-based CSPs. Early elution and poor or no enantioseparation can result under these conditions. Acidic mobile phase additives are often required to suppress the dissociation of acidic analytes, resulting in increased retention and improved enantioselectivity.

Three volatile organic acids, (trifluoroacetic acid (TFA, p$K_a$ 0.59), formic acid (FA, p$K_a$ 3.75), and acetic acid (HAc, p$K_a$ 4.76) were evaluated on Lux® Cellulose-1 and Lux Amylose-2 CSPs as acidic additives. In general, these additives provide similar enantioresolution for weakly acidic racemates (**Figures 3A-3C**) while the stronger acidic additive (TFA) performs better for the stronger acidic racemates (**Figures 3D–3F**). Formic acid provides comparable enantioseparations and peak shapes to TFA. Considering the "memory effect" of TFA commonly experienced on polysaccharide-based CSPs and its ion suppressing tendency in MS detection, formic acid was selected in preference over TFA in reversed phase mobile phase for the chiral separation and MS/MS detection of acidic racemates.

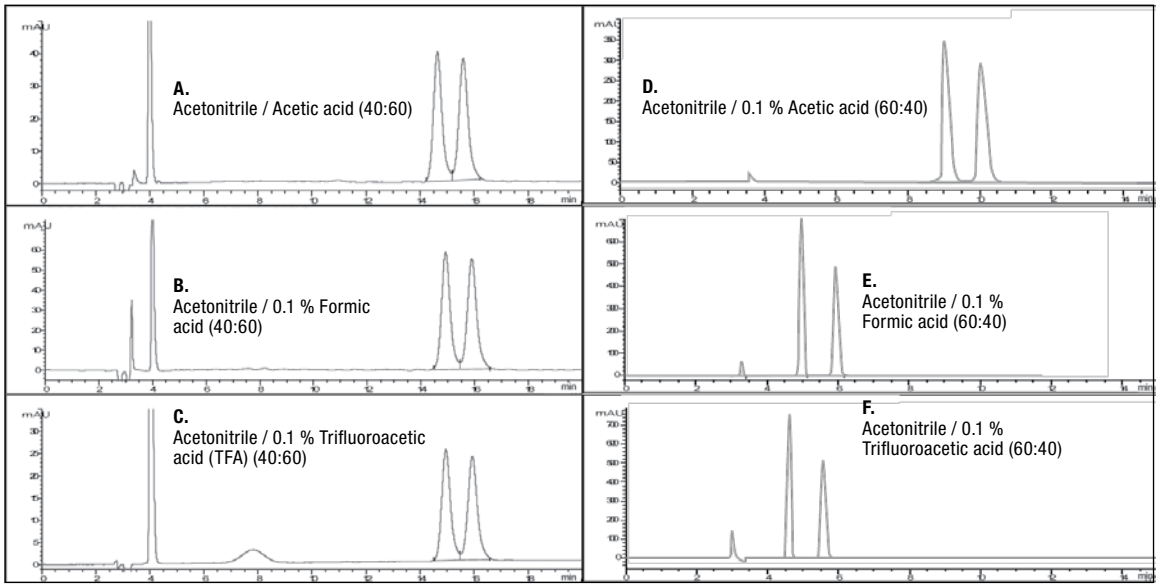
*Effect of Mobile Phase Additives*
The LC/MS/MS responses of acidic racemates with ESI negative (ESI-) mode in 5 mM ammonium formate, 5 mM ammonium acetate, and 5 mM ammonium bicarbonate containing mobile phases with either acetonitrile or methanol as organic modifier using an achiral column (Kinetex® 2.6 µm C18) were compared to responses in 0.1% formic acid **(Figures 4 and 5)**. The results show that MS/MS responses using 0.1% formic acid in acetonitrile or methanol are comparable to the responses using the other acid additives in ESI- mode, with the exception of carprofen which showed poor response with all of the acidic mobile phase additives. This shows that using 0.1 % formic acid as acidic mobile phase additive is fully compatible with MS/MS detection and can be implemented as the first choice for mobile phase additive.

*Effect of Organic Modifier on Chiral Resolution*
Acetonitrile or methanol was the organic modifier used in chiral reversed phase HPLC. Decreasing the eluting strength of the mobile phase by decreasing the percentage of acetonitrile or methanol in the mobile phase will increase retention and resolution (**Figure 6**). However, once enantiomers elute later than about 10 minutes with only partial resolution, baseline separation can be rarely achieved by further decreasing the % organic modifier in the mobile phase. In our study, acetonitrile was more successful than methanol in providing chiral resolution on Lux CSPs in reversed phase (RP) mode (**Figure 8**), typically yielding sharper and narrower peaks.

**Figure 3.**
Effect of Acidic Additives on the Enantioseparation of Acidic Racemates in Reversed Phase

**Bendroflumethiaze**
Lux® 5 µm Cellulose-1
Part Number: 00G-4458-E0

**Flurbiprofen**
Lux 5 µm Amylose-2
Part Number: 00G-4471-E0

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**Column:** As noted
**Dimensions:** 250 x 4.6 mm
**Flow Rate:** 1 mL/min
**Temperature:** 25 °C
**Detection:** UV @ 220 nm
**Injection Volume:** 10 µL
**Mobile Phase:** As noted

**Figure 4.**
LC/MS/MS Responses of Acidic Racemates in ESI- with Different Additives and Methanol

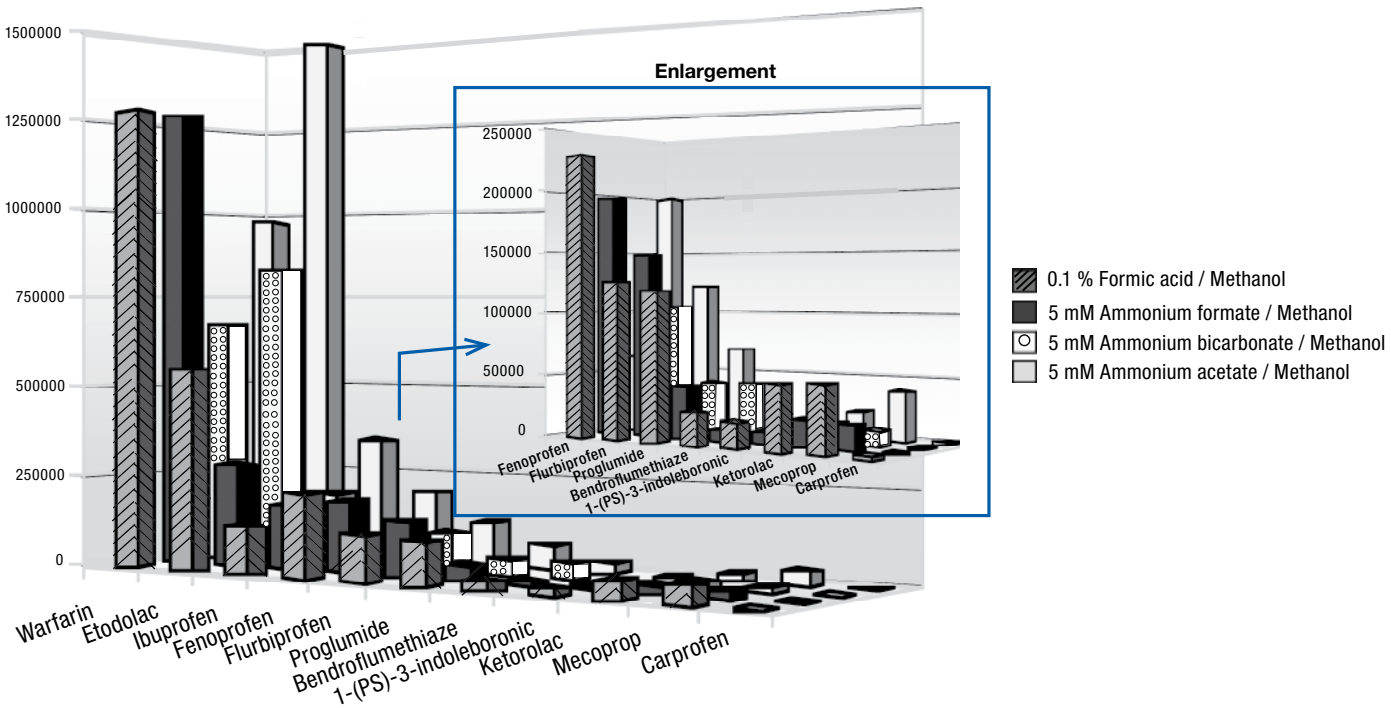


**Figure 5.**
LC/MS/MS Responses of Acidic Racemates in ESI- with Different Additives and Methanol

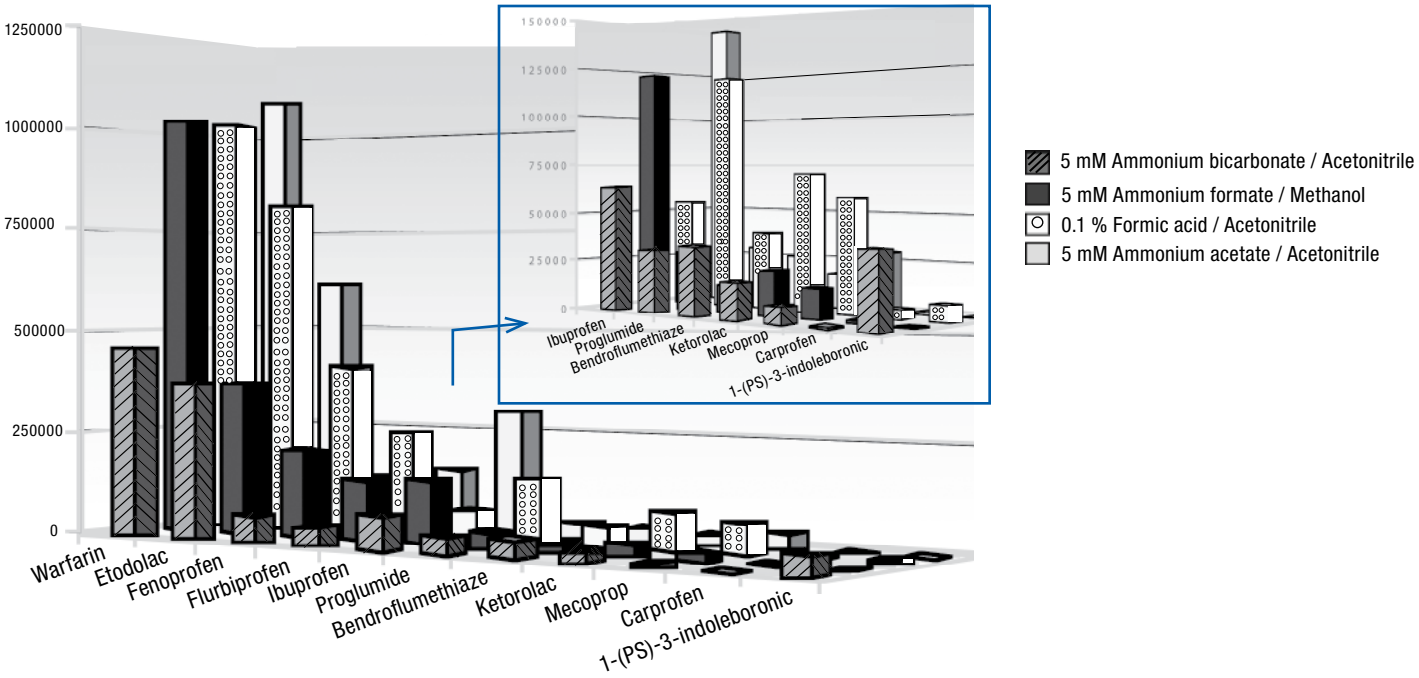


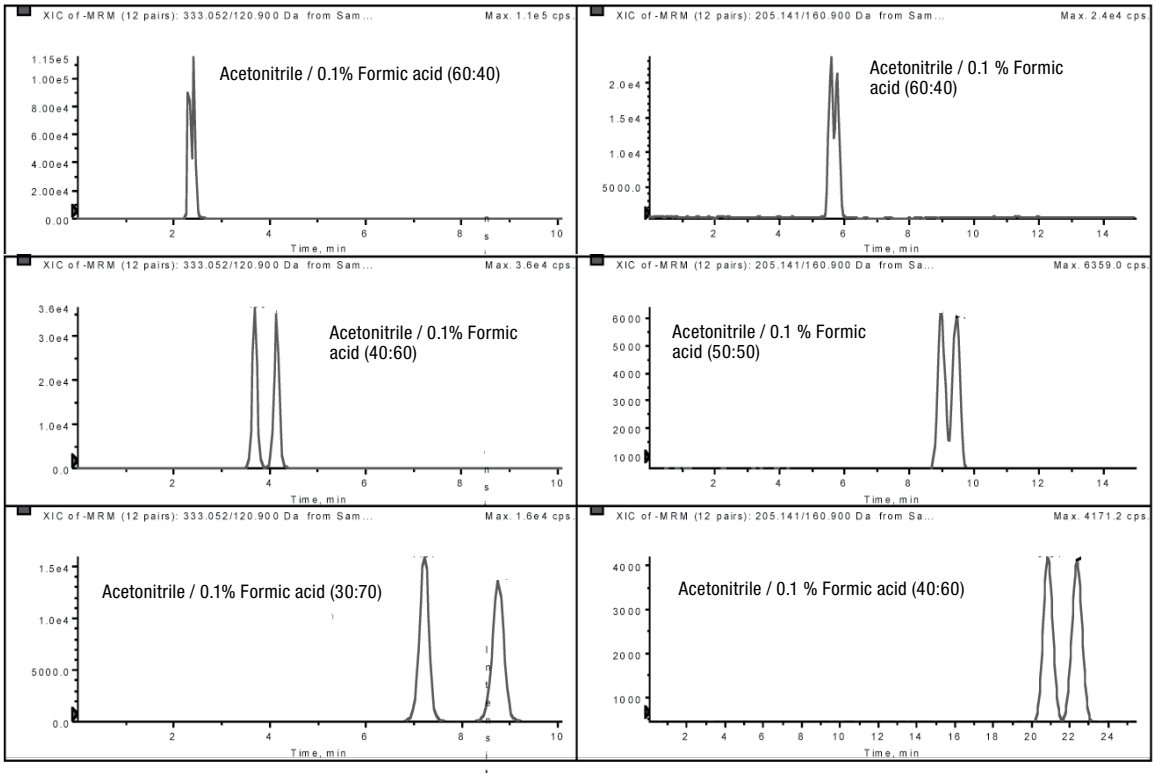

**Figure 6.**
Effect of Acidic Additives on the Enantioseparation of Acidic Racemates in Reversed Phase

**Proglumide**

Lux® 3 µm Cellulose-1
Flow Rate: 0.2 mL/min
Dimensions: 150 x 2.0 mm

**Ibuprofen**

Lux 5 µm Cellulose-3
Flow Rate: 1 mL/min
Dimensions: 250 x 4.6 mm




**Column:** As noted
**Dimensions:** As noted
**Flow Rate:** As noted
**Temperature:** 25 °C
**Detection:** Mass Spectrometer (MS)
**Injection Volume:** 5-20 µL
**Mobile Phase:** As noted
**Sample:** As noted

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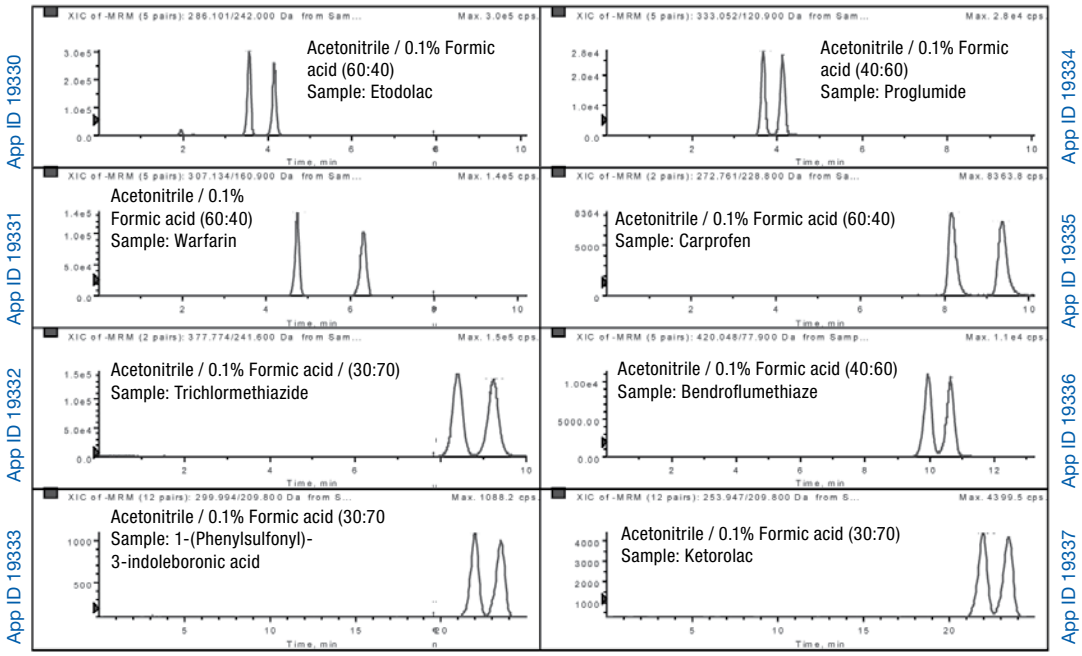
App ID 19329

# APPLICATIONS

*Chiral LC/MS/MS Applications*

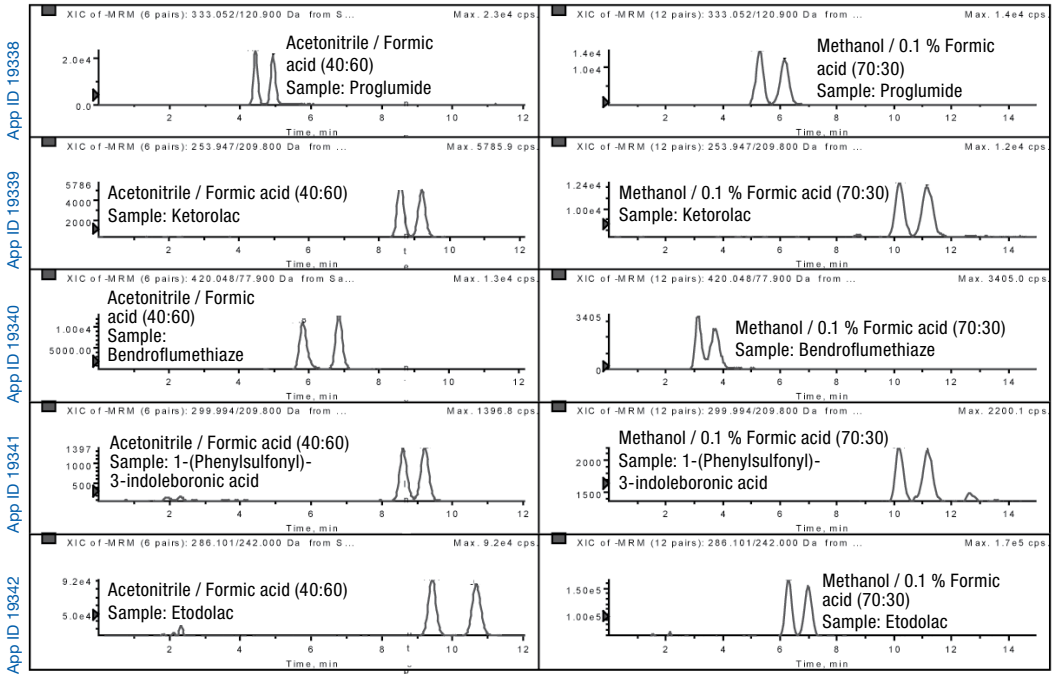
**Figures 7-11** demonstrate 15 chiral separations on several different Lux chiral stationary phases. APCI negative (APCI-) mode was employed for the MS/MS detection of carprofen, abscisic acid, and fenoprofen as it provides much better MS/MS signals than ESI- for these acidic racemates. ESI positive (ESI+) mode was used for the MS/MS detection of suprofen and gave slightly improved signal intensity for warfarin and ketorolac versus ESI- (**Figure 10**). Most compounds evaluated here eluted in less than 10 min with baseline resolution in mobile phases of various elution strength. The results show that Lux® Cellulose-3 was most successful in separating acidic racemates (ten racemates), especially nonsteroidal anti-inflammatory drugs.

**Figure 7.**
Enantioseparations in Reversed Phase on Lux 3 µm Cellulose-1 with Acetonitrile



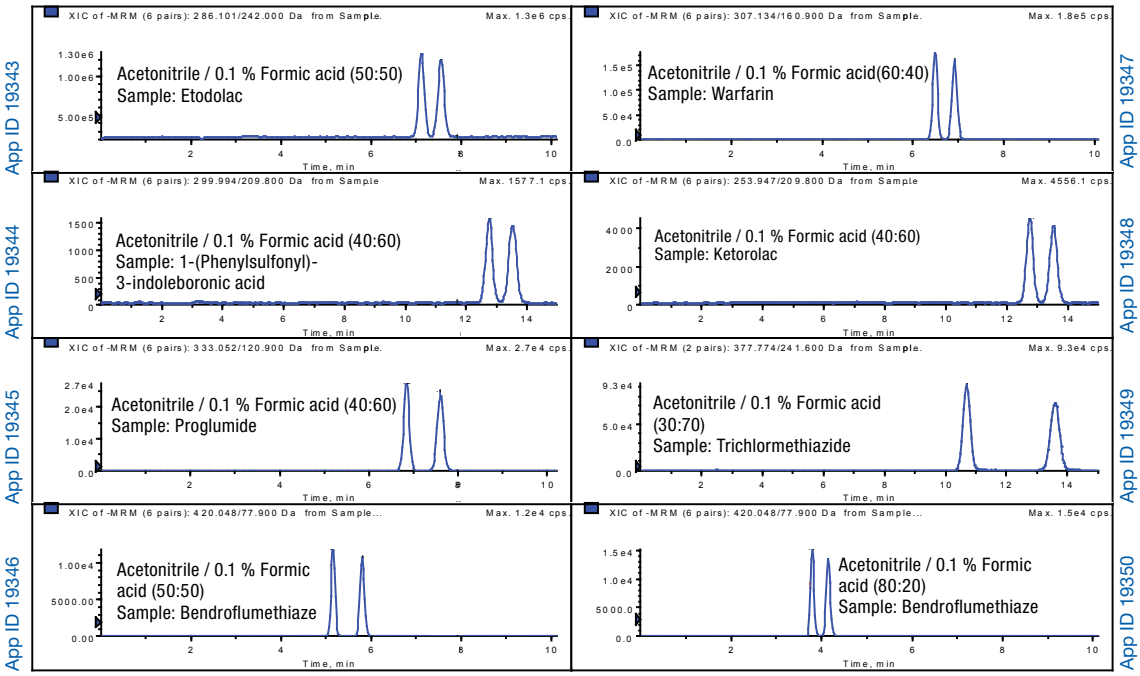

**Column:** Lux 3 µm Cellulose-1
**Dimensions:** 150 x 2.0 mm
**Part No.:** 00F-4458-B0
**Flow Rate:** 0.2 mL/min
**Temperature:** 25 °C
**Detection:** Mass Spectrometer (MS)
**Injection Volume:** 5 µL
**Mobile Phase:** As noted
**Sample:** As noted

**Figure 8.**
Enantioseparations on Lux 3 µm Cellulose-2 with Acetonitrile or Methanol as Modifier



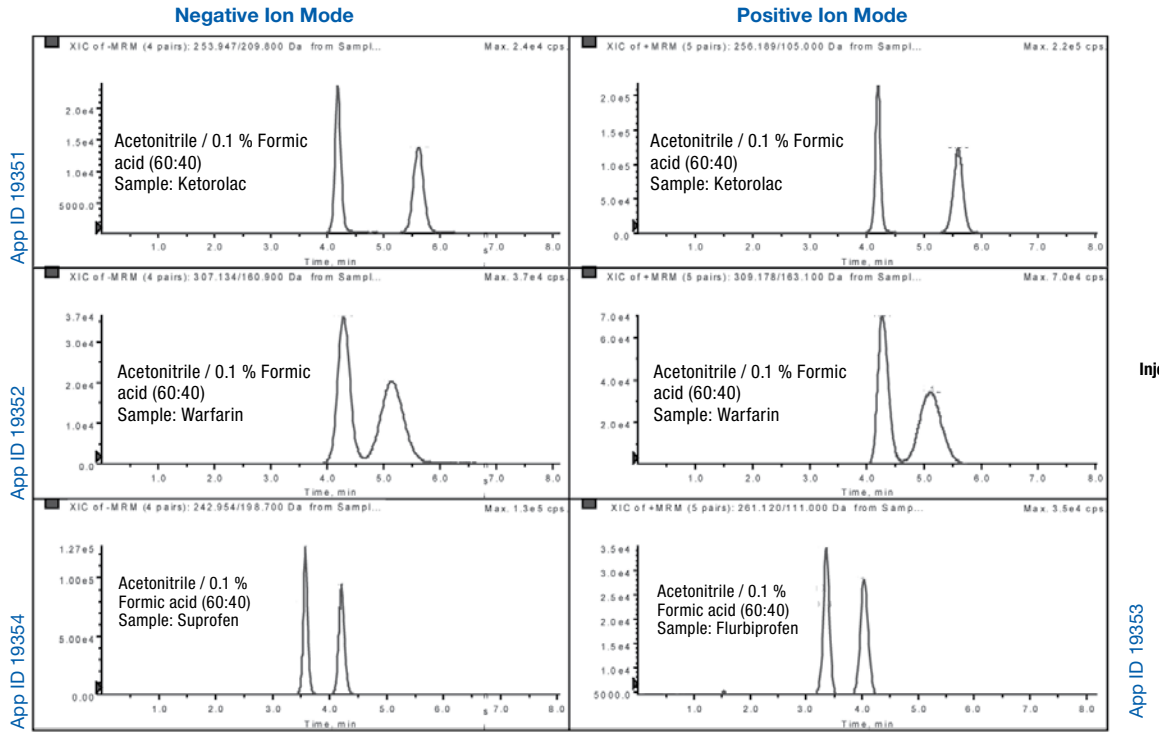

**Column:** Lux 3 µm Cellulose-2
**Dimensions:** 150 x 2.0 mm
**Part No.:** 00F-4456-B0
**Flow Rate:** 0.2 mL/min
**Temperature:** 25 °C
**Detection:** Mass Spectrometer (MS)
**Injection Volume:** 5 µL
**Mobile Phase:** As noted
**Sample:** As noted

**Figure 9.**
Enantioseparations on Lux® 5 µm Cellulose-4 with Acetonitrile



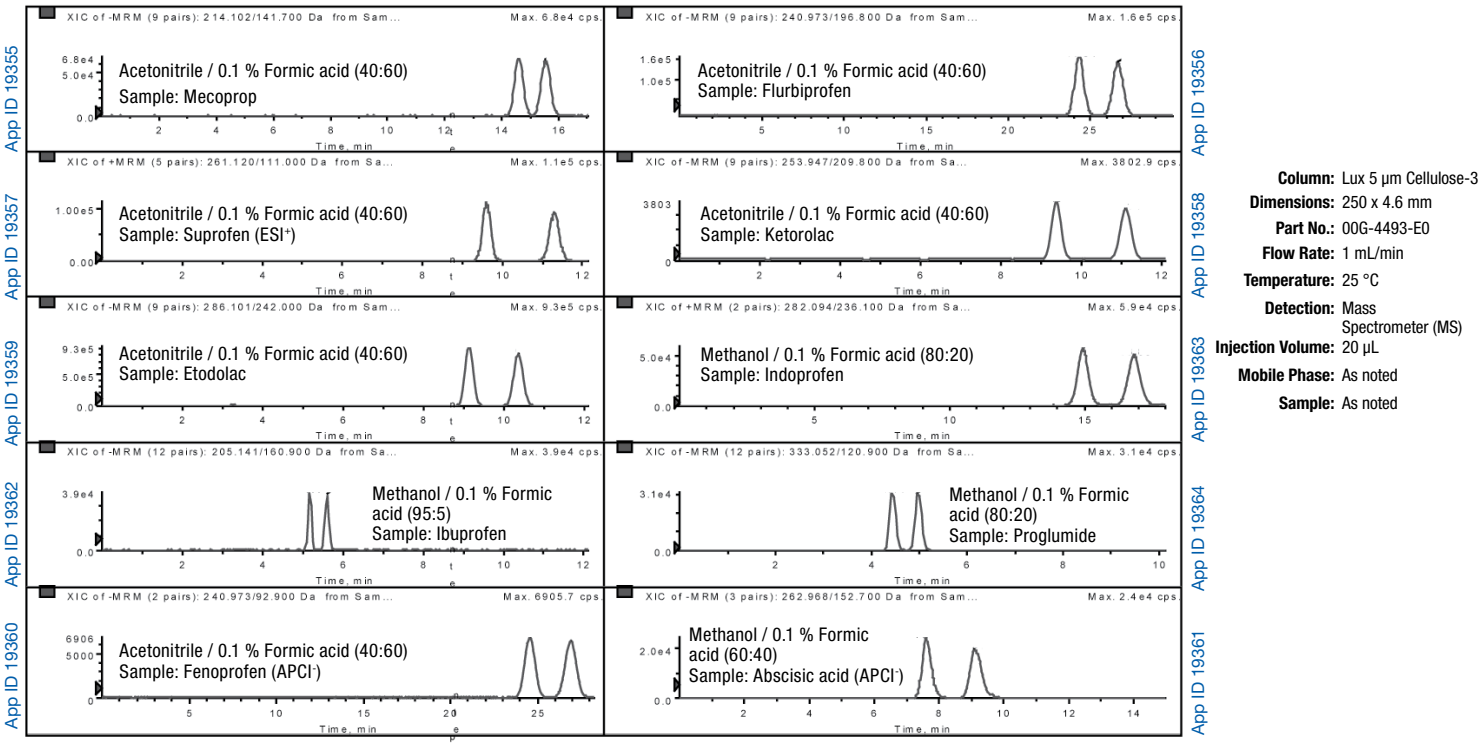

**Column:** Lux 5 µm Cellulose-4
**Dimensions:** 250 x 4.6 mm
**Part No.:** 00G-4491-E0
**Flow Rate:** 1 mL/min
**Temperature:** 25 °C
**Detection:** Mass Spectrometer (MS)
**Injection Volume:** 20 µL
**Mobile Phase:** As noted
**Sample:** As noted

**Figure 10.**
Enantioseparations in Reversed Phase on Lux 3 µm Amylose-2 with Acetonitrile




**Column:** Lux 3 µm Amylose-2
**Dimensions:** 150 x 2.0 mm
**Part No.:** 00F-4471-B0
**Flow Rate:** 0.2 mL/min
**Temperature:** 25 °C
**Detection:** Mass Spectrometer (MS)
**Injection Volume:** 5 µL
**Mobile Phase:** As noted
**Sample:** As noted

**Figure 11.**
Enantioseparations in Reversed Phase on Lux® 5 µm Cellulose-3 with Acetonitrile or Methanol as Modifier




**Column:** Lux 5 µm Cellulose-3
**Dimensions:** 250 x 4.6 mm
**Part No.:** 00G-4493-E0
**Flow Rate:** 1 mL/min
**Temperature:** 25 °C
**Detection:** Mass Spectrometer (MS)
**Injection Volume:** 20 µL
**Mobile Phase:** As noted
**Sample:** As noted

## Conclusions

The chiral LC/MS/MS analysis of fifteen different acidic racemates are successfully demonstrated on the polysaccharide-based CSPs Lux Cellulose-1, Lux Cellulose-2, Lux Cellulose-3, Lux Cellulose-4, and Lux Amylose-2 in reversed phase (RP) elution mode.

Formic acid is a good first choice for an acidic RP mobile phase additive as it leads to increased retention and improved enantioselectivity for acidic enantiomers and is also compatible with MS/MS detection.

Increasing the percentage of organic modifier (acetonitrile or methanol) in the RP mobile phase has the expected effect of decreasing retention and enantioselectivity. Adjusting the organic modifier content of the mobile phase is therefore essential to optimizing chiral resolution.

# APPLICATIONS

## Lux® Ordering Information

### 3 µm Analytical Columns (mm)

| | 50 x 2.0 | 150 x 2.0 | 50 x 4.6 | 100 x 4.6 | 150 x 4.6 | 250 x 4.6 | SecurityGuard™ Cartridges (mm) 4 x 2.0* | 4 x 3.0* |
|---|---|---|---|---|---|---|---|---|
| Phases | | | | | | | /10pk | /10pk |
| **Cellulose-4** | 00B-4490-B0 | 00F-4490-B0 | 00B-4490-E0 | 00D-4490-E0 | 00F-4490-E0 | 00G-4490-E0 | AJ0-8626 | AJ0-8627 |
| **Cellulose-3** | 00B-4492-B0 | 00F-4492-B0 | 00B-4492-E0 | 00D-4492-E0 | 00F-4492-E0 | 00G-4492-E0 | AJ0-8621 | AJ0-8622 |
| **Cellulose-2** | 00B-4456-B0 | 00F-4456-B0 | 00B-4456-E0 | 00D-4456-E0 | 00F-4456-E0 | 00G-4456-E0 | AJ0-8398 | AJ0-8366 |
| **Cellulose-1** | 00B-4458-B0 | 00F-4458-B0 | 00B-4458-E0 | 00D-4458-E0 | 00F-4458-E0 | 00G-4458-E0 | AJ0-8402 | AJ0-8403 |
| **Amylose-2** | 00B-4471-B0 | 00F-4471-B0 | 00B-4471-E0 | 00D-4471-E0 | 00F-4471-E0 | 00G-4471-E0 | AJ0-8471 | AJ0-8470 |
| | | | | | | for ID: | 2.0–3.0 mm | 3.2–8.0 mm |

### 5 µm Analytical Columns (mm)

| | 50 x 2.0 | 50 x 4.6 | 100 x 4.6 | 150 x 4.6 | 250 x 4.6 | SecurityGuard™ Cartridges (mm) 4 x 2.0* | 4 x 3.0* |
|---|---|---|---|---|---|---|---|
| Phases | | | | | | /10pk | /10pk |
| **Cellulose-4** | 00B-4491-B0 | 00B-4491-E0 | 00D-4491-E0 | 00F-4491-E0 | 00G-4491-E0 | AJ0-8626 | AJ0-8627 |
| **Cellulose-3** | 00B-4493-B0 | 00B-4493-E0 | 00D-4493-E0 | 00F-4493-E0 | 00G-4493-E0 | AJ0-8621 | AJ0-8622 |
| **Cellulose-2** | 00B-4457-B0 | 00B-4457-E0 | 00D-4457-E0 | 00F-4457-E0 | 00G-4457-E0 | AJ0-8398 | AJ0-8366 |
| **Cellulose-1** | 00B-4459-B0 | 00B-4459-E0 | 00D-4459-E0 | 00F-4459-E0 | 00G-4459-E0 | AJ0-8402 | AJ0-8403 |
| **Amylose-2** | 00B-4472-B0 | 00B-4472-E0 | 00D-4472-E0 | 00F-4472-E0 | 00G-4472-E0 | AJ0-8471 | AJ0-8470 |
| | | | | | for ID: | 2.0–3.0 mm | 3.2–8.0 mm |

### 5 µm Semi-Prep Columns (mm)

| | 150 x 10.0 | 250 x 10.0 | SecurityGuard™ Cartridges (mm) 10 x 10.0‡ |
|---|---|---|---|
| Phases | | | /3pk |
| **Cellulose-4** | 00F-4491-N0 | 00G-4491-N0 | AJ0-8628 |
| **Cellulose-3** | 00F-4493-N0 | 00G-4493-N0 | AJ0-8623 |
| **Cellulose-2** | 00F-4457-N0 | 00G-4457-N0 | AJ0-8399 |
| **Cellulose-1** | 00F-4459-N0 | 00G-4459-N0 | AJ0-8404 |
| **Amylose-2** | 00F-4472-N0 | 00G-4472-N0 | AJ0-8472 |
| | | for ID: | 9-16 mm |

### 20 µm Bulk Media

| | 100 g | 1 kg |
|---|---|---|
| Phases | | |
| **Cellulose-1** | 04G-4473 | 04K-4473 |
| **Cellulose-2** | 04G-4464 | 04K-4464 |
| **Cellulose-3** | 04G-4504 | 04K-4504 |
| **Cellulose-4** | 04G-4503 | 04K-4503 |

### 5 µm Axia™ Packed Preparative Columns (mm)

| | 150 x 21.2 | 250 x 21.2 | 250 x 30 | 250 x 50 | SecurityGuard™ Cartridges (mm) 15 x 21.2** | 15 x 30.0+ |
|---|---|---|---|---|---|---|
| Phases | | | | | /ea | /ea |
| **Cellulose-4** | 00F-4491-P0-AX | 00G-4491-P0-AX | 00G-4491-U0-AX | 00G-4491-V0-AX | AJ0-8629 | AJ0-8630 |
| **Cellulose-3** | 00F-4493-P0-AX | 00G-4493-P0-AX | 00G-4493-U0-AX | 00G-4493-V0-AX | AJ0-8624 | AJ0-8625 |
| **Cellulose-2** | 00F-4457-P0-AX | 00G-4457-P0-AX | 00G-4457-U0-AX | 00G-4457-V0-AX | AJ0-8400 | AJ0-8401 |
| **Cellulose-1** | 00F-4459-P0-AX | 00G-4459-P0-AX | 00G-4459-U0-AX | 00G-4459-V0-AX | AJ0-8405 | AJ0-8406 |
| **Amylose-2** | 00F-4472-P0-AX | 00G-4472-P0-AX | 00G-4472-U0-AX | 00G-4472-V0-AX | AJ0-8473 | AJ0-8474 |
| | | | | for ID: | 18–29 mm | 30–49 mm |

*SecurityGuard Analytical Cartridges require holder, Part No. : KJ0-4282
‡SemiPrep SecurityGuard Cartridges require holder, Part No. : AJ0-7220
**PREP SecurityGuard Cartridges require holder, Part No. : AJ0-8223
+PREP SecurityGuard Cartridges require holder, Part No. : AJ0-8277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guarantee

If Lux analytical columns (≤ 4.6 mm ID) do not provide at least an equivalent or better separation as compared to a competing column of the same particle size, similar phase and dimensions, return the column with comparative data within 45 days for a FULL REFUND.



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