

# **Identification, Isolation and Stability Studies of Atropisomers in Drug Discovery Projects**

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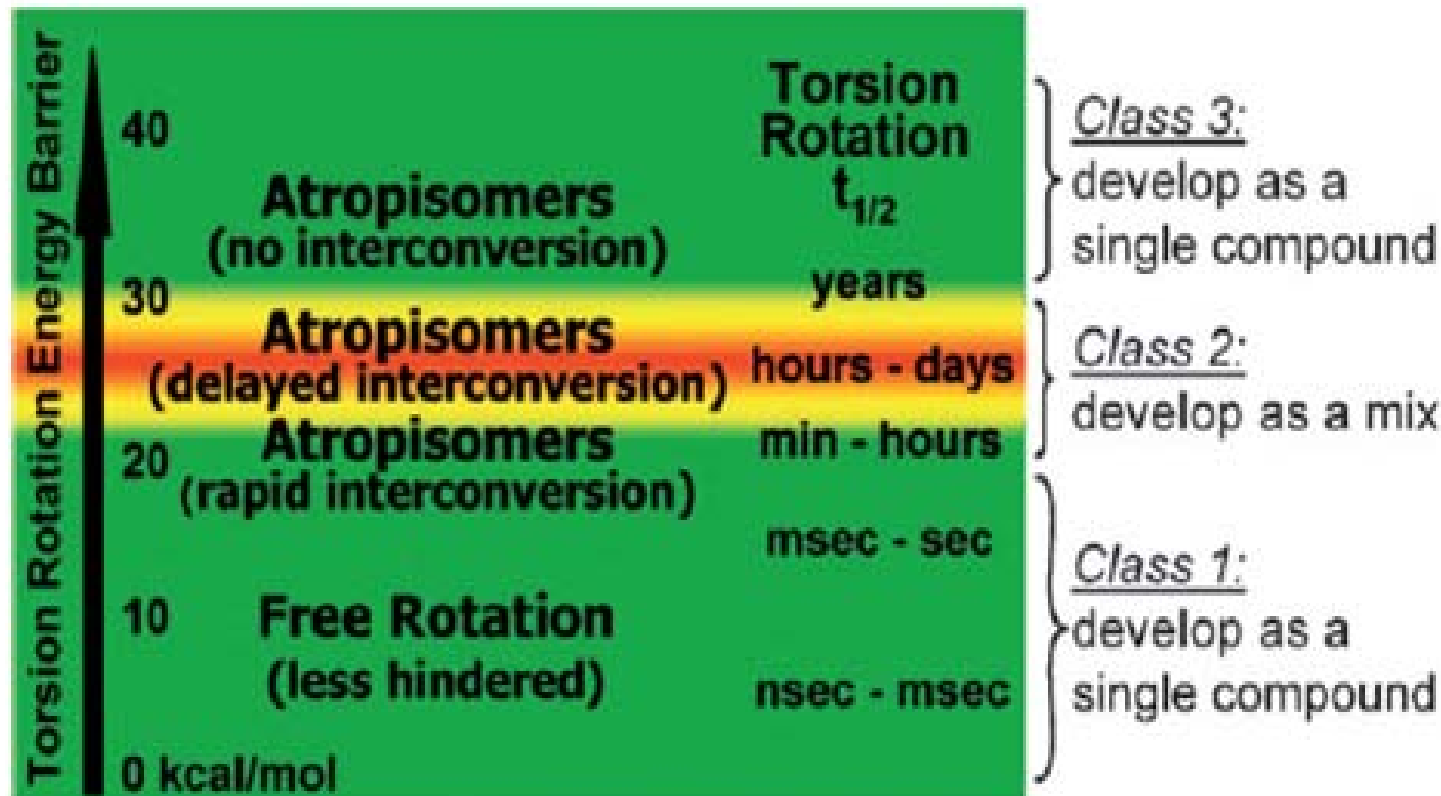
# Outline

- **Introduction on Atropisomers**
- **Identification of Atropisomers**
- **Isolation for toxicity and stability studies**
- **Analytical support in thermal conversion study**
- **Analytical support in In-Vitro stability study**
- **Absolute configuration study**
- **Conclusion**

# What is an Atropisomer

- Most chiral molecules have a chiral center
- Atropisomer has a rotational axis with a hindered rotational barrier
- Atropisomers are recognized as separable species when at a given temperature they display a half life of at least 1,000 seconds
- This axial rotational constraint results in chirality termed atropisomerism (from the Greek, *a*= not and *tropos*= turn)

# Regulatory Guidance on Atropisomerism



# Configuration of Atropisomers

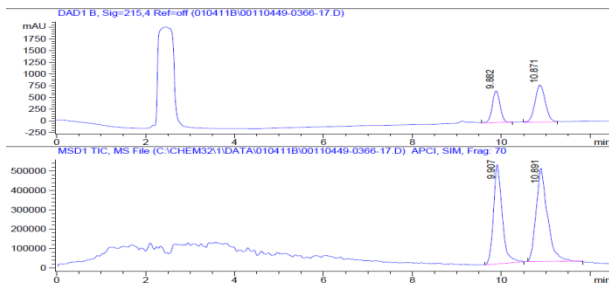
- The substituents are assigned according to drawing the shortest 90 degree path from the substituent of the highest priority on the proximal ring to the highest ranked substituent on the distal ring. If the 90 degree turn is clockwise the absolute configuration is P; if counterclockwise the absolute configuration is M.

# Identification of Atropisomers

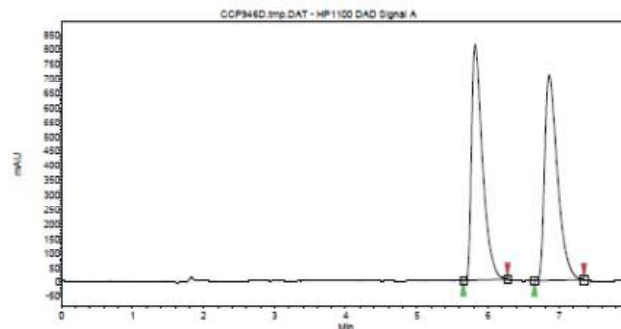
- Atropisomers with a certain energy barrier at a given temperature can be resolved by chiral chromatography, crystallization, or resolution
- The chromatographic separation is very much dependent on the temperature
- The chromatographic separation is also dependent on the substituents on the biaryl rings

# Atropisomers in Drug Discovery Projects

Several atropisomers were identified in various discovery projects with SFC or HPLC methods

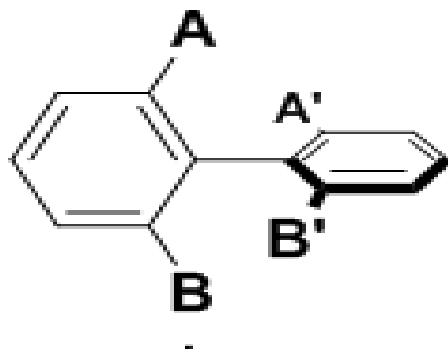


HPLC/MS method: cellulose-3,  
4.6x250mm, 5 $\mu$ , 5% ethanol linear  
gradient to 95% ethanol in heptane in  
10mins, 1.5ml/min



SFC method: ChiralPAK-AD-H,  
4.6x250mm, 5 $\mu$ , 25% methanol,  
75% CO<sub>2</sub>, 2.5ml/min, 35°C, 120  
bars

# Chromatographic Resolutions of Atropisomers

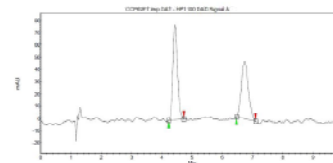


The substituents on the biphenyl system (A, A', B, B') play very important role for the chromatographic resolution on atropisomers. The resolution is dependent on:

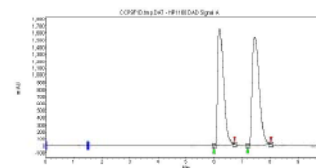
1. the numbers of the substituted functional groups
2. the size of substituted functional groups
3. the distance of substituted functional groups
4. the column temperature

# Analytical Separations vs. Numbers of Substituted Functional Group

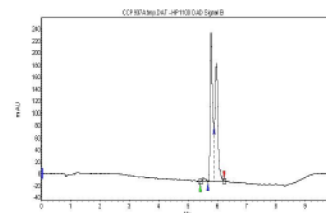
With 3-4 substituents: great



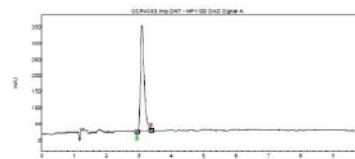
With 2 substituents: good



With 1 substituent: minor

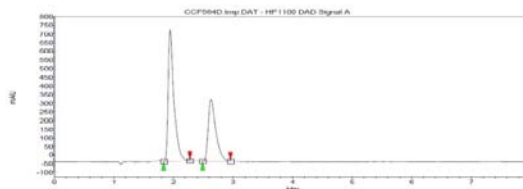


With 0 substituent: no

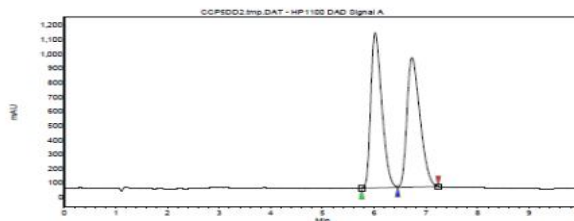


# Analytical Separations vs. Size of Substituted Functional Groups

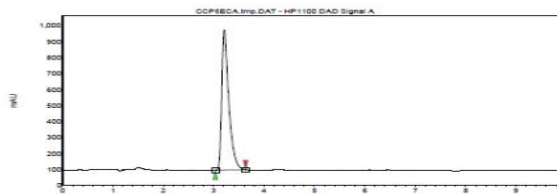
Methoxy group substituent



Methyl group substituent



Fluorine group substituent

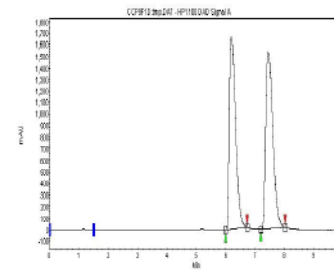


***Fluorine substituent in the ortho positions could have low rotational barrier, in some cases, no separations were evident on SFC chromatography***

# Analytical Separations vs. Distance of Substituted Functional Groups

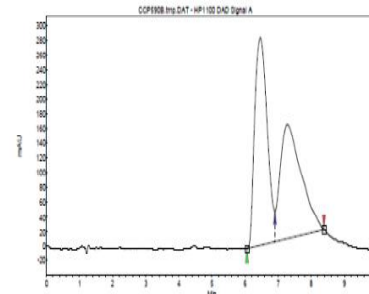
A,B and A',B' next each other:

good resolution



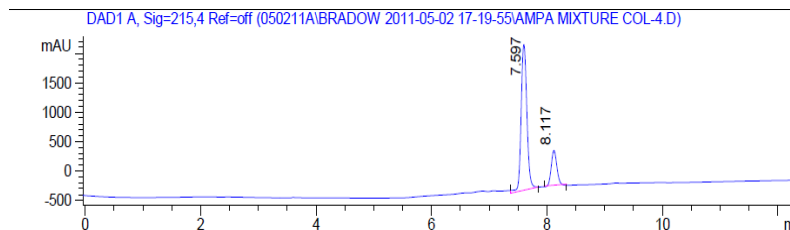
A,B and A',B' away each other:

less resolution

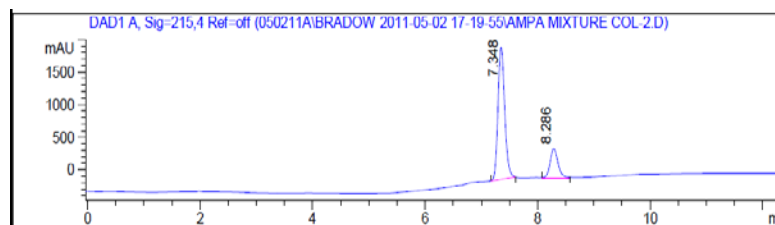


# Analytical Separations vs. Column Temperature

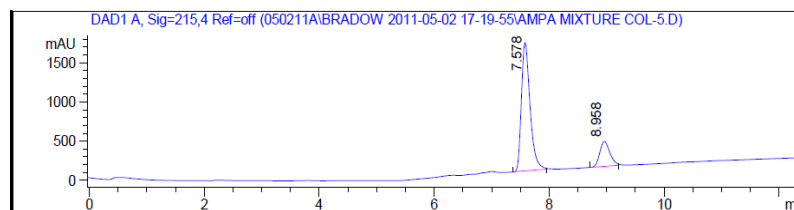
Column temperature 40°C



Column temperature 25°C



Column temperature 10°C



***Lowering the column temperature may slow down the rotation and also affect the interactions between the molecules and stationary phase, resulting in increased selectivity***

# **Comparisons of SFC Method vs. HPLC Method for Atropisomers**

- SFC method has short elution time, better selectivity in most cases, and also saves organic solvents
- HPLC method allows to runs at wide range of column temperatures, it could be crucial to see the separations in some cases
- HPLC/MS has better detection sensitivity, allows to analyze samples at low concentration

# Analytical Separations on an Early Lead Compound

Separation of atropisomer for an early lead compound was evident on several chiral columns. Best selectivity was obtained on ChiralPAK-AD-H with 25% methanol as co-solvent

Columns	Co-solvent	Capacity factor	Selectivity
<b>ChiralPAK-AD-H</b>	<b>25% methanol</b>	<b>4.82</b>	<b>1.28</b>
ChrialPAK-AS-H	25% methanol	5.20	1.20
Chiralcel-OJ-H	25% methanol	5.14	1.08
ChrialPAK-IA	20% methanol	4.22	1.15

# Isolation of Atropisomers

- Atropisomers were isolated on the preparative SFC columns for various tox. studies
- For the early lead compound, Berger Multigram-II SFC system was used on ChiralPAK-AD-H, 21x250mm, with 25% methanol as a modifier
- Peak-2 was the desired isomer
- Several batches (~2g-5g for each batch) were purified

	Purity	yield
Peak1	~98.5%	~91%
Peak2	~97%	~88%

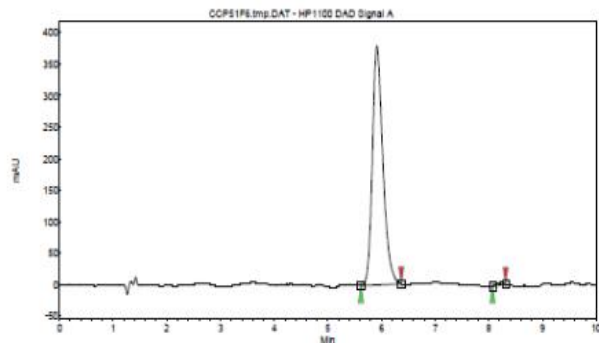
# Toxicity Results on Isolated Isomers

- At the receptor, one atropoenantiomers was found to be approximately 4X more potent than the other atropoenantiomers
- In-vivo profiling of both atropoenantiomers revealed one atropoenantiomers to be inactive in our locomotor assay at 32 mg/kg while the more potent atropoenantiomers was active in doses as low as 5 mg/kg

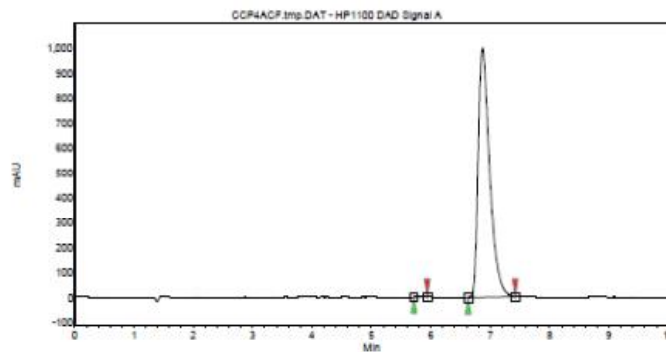
	atropoenantiomers-1	atropoenantiomers-2	racemate
Functions	5nM	19nM	14nM
Binding	5nM	17nM	12nM
THLF	17uM	101uM	73uM

# Ultrapure Material for Stability Studies

- Ultrapure atropoenantiomers are required to perform the atropisomer interconversion kinetics studies
- Ultrapure material was obtained by multiple purifications on the SFC chiral columns (AD column and AS column) for the crude isomer
- >99% ee atropoenantiomer was provided to the project team



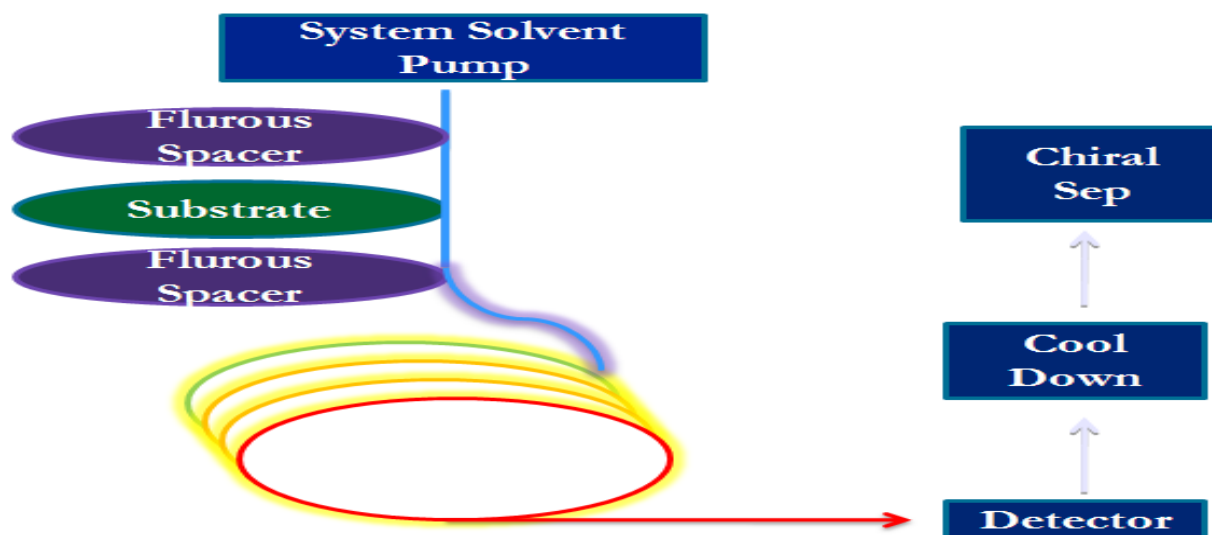
Atropoenantiomer-1



Atropoenantiomer-2

# Interconversion Studies

- Interconversion studies were conducted on a segmented flow reactor in ROC laboratory



The solution was heated in a segmented flow reactor to four different temperatures and sampled in duplicate at 11 predetermined intervals and analyzed by SFC chiral chromatography to determine % *ee*

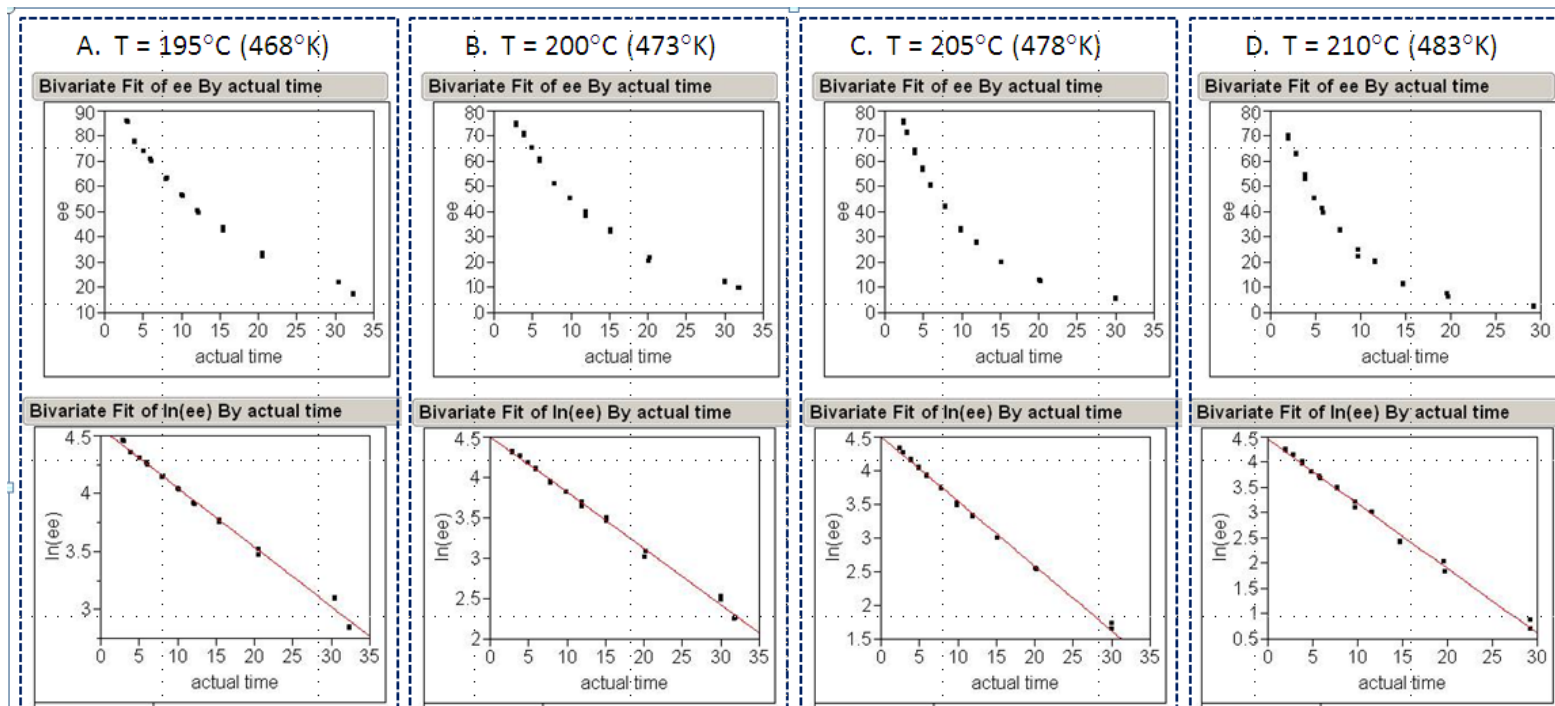
# Analytical Results for Stability Studies

number	notebook#	design time (min)	reaction temperature	set time (min)	actual time			ee
						%Area Peak1	%Area Peak2	
1	00110449-0368-001	2.5	205 C	2.8	2.5	12.2	87.8	75.6
2	00110449-0368-002	2.5	205 C	2.8	2.5	12.5	87.5	75.0
3	00110449-0368-003	3	205 C	3.6	3.01	14.4	85.6	71.2
4	00110449-0368-004	3	205 C	3.6	3.00	14.5	85.5	71.0
5	00110449-0368-005	4	205 C	4.6	3.98	17.9	82.1	64.2
6	00110449-0368-006	4	205 C	4.6	3.97	18.5	81.5	63.0
7	00110449-0368-007	5	205 C	5.8	5.03	21.9	78.1	56.2
8	00110449-0368-008	5	205 C	5.8	5.00	21.5	78.5	57.0
9	00110449-0368-009	6	205 C	6.9	6.01	25.1	74.9	49.8
10	00110449-0368-010	6	205 C	6.9	6.03	24.7	75.3	50.6
11	00110449-0368-011	8	205 C	9.2	8.02	29.2	70.8	41.6
12	00110449-0368-012	8	205 C	9.2	8.02	29	71	42.0
13	00110449-0368-013	10	205 C	11.5	10.05	33.4	66.6	33.2
14	00110449-0368-014	10	205 C	11.5	10.04	33.9	66.1	32.2
15	00110449-0368-015	12	205 C	13.8	12.03	36.3	63.7	27.4
16	00110449-0368-016	12	205 C	13.8	12.02	36.2	63.8	27.6
17	00110449-0368-017	15	205 C	17.3	15.18	40	60	20.0
18	00110449-0368-018	15	205 C	17.3	15.20	40.1	59.9	19.8
19	00110449-0368-019	20	205 C	23	20.33	43.8	56.2	12.4
20	00110449-0368-020	20	205 C	23	20.23	43.6	56.4	12.8
21	00110449-0368-021	30	205 C	34.5	30.15	47.2	52.8	5.6
22	00110449-0368-022	30	205 C	34.5	30.08	47.4	52.6	5.2
23	00110449-0368-023	15	40 C	15 (control)	16.94	1.3	98.7	97.4
24	00110449-0368-024	15	40C	15 (control)	16.93	0.4	99.6	99.2
25	00110449-0368-025	starting material				1.2	98.8	97.6

# Interconversion Studies

- At the start of the reaction, some of atropisomer A is converted to a high energy planar intermediate.
- The planar intermediate can then convert back to starting atropisomer A (**1**) or into B (**2**) resulting in a static mixture at equilibrium.

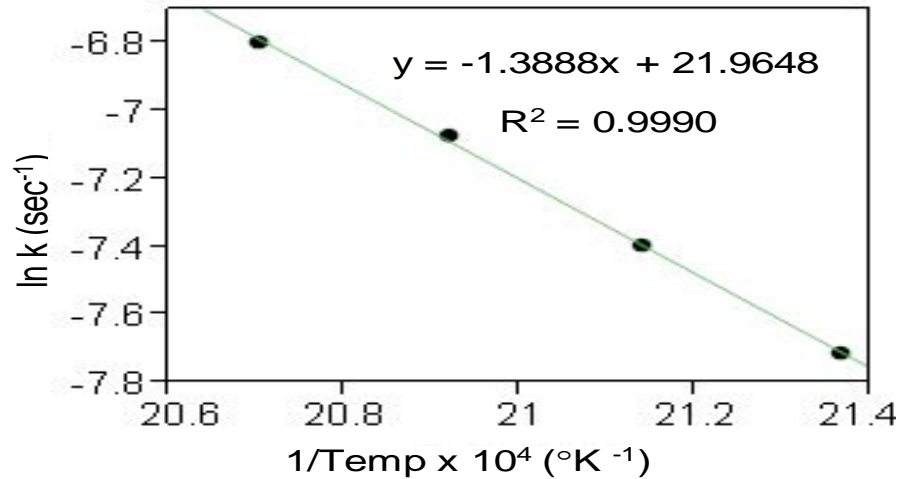
# Interconversion Kinetics



a first order rate equation can be used to approximate the relationship of atropisomer A to B with respect to time.

where  $x$  represents the amount of A converted to B at time  $t$  and  $[x]_e$  is  $[x]$  at equilibrium. In the case of atropoenantiomers, where the two products are of equal energy, equilibrium results in a 1:1 racemic mixture (0% *ee*) of atropoenantiomers A to B.

# Half life of Atropisomer



Ea is calculated to be 27.6  
kcal/mol

Rate (2k) @ 25 °C (298 K) =  
2.39E-9 min<sup>-1</sup>

t<sub>1/2</sub> = ln 2/k, where k = k<sub>f</sub> + k<sub>b</sub>

t<sub>1/2</sub> at 25 °C- 558 years

t<sub>1/2</sub> at 37 °C- 89 years

# Interconversion Kinetics in Protein Containing Media

- Human blood, human plasma, and human serum albumin were chosen to study the effect of proteins on interconversion. Phosphate buffer saline was used as a control
- Assuming that the drug will be effective at a low concentration compared to the concentration of serum protein in the physiological matrix, the racemate and atropoenantiomers were individually incubated at 37°C at a concentration of 0.1 mM for each matrix at a neutral pH of 7.3
- Samples were maintained at 37°C and extracted into acetonitrile at defined time points up to 24 hours and analyzed on HPLC/MS

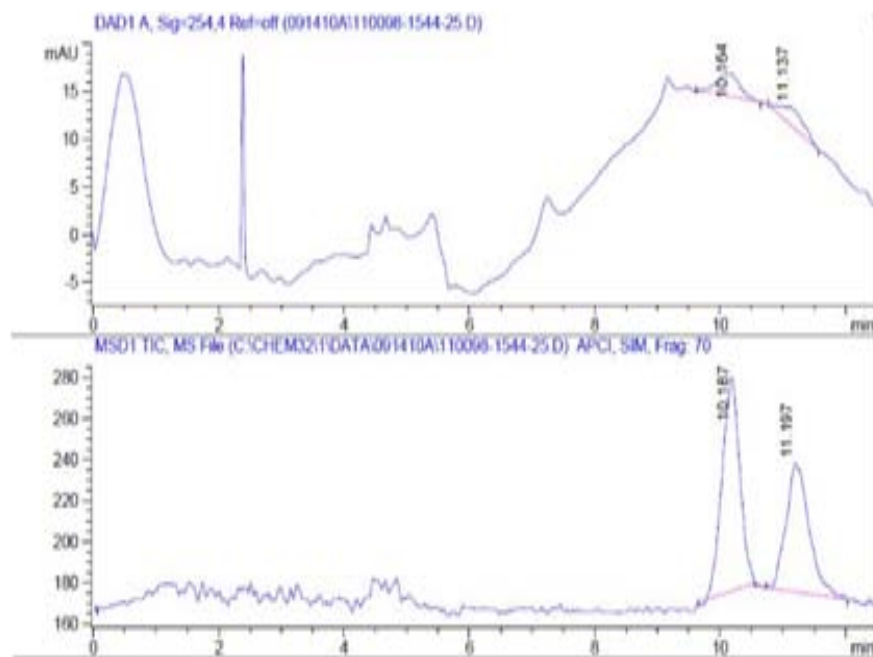
# Analytical Method Development For Low Concentration Samples

- Sample concentration estimated at 38ug/ml
- Current SFC chiral method is not suitable for this purpose, detection limits on SFC chiral method is estimated ~50ug/ml
- A new HPLC with mass and UV detectors are used for this analysis
- Analytical HPLC method was forwarded to PDM department

# Analytical Conditions for HPLC/MS

- Column: Cellulose-3, 5 $\mu$ , 4.6mmx250mm
- Mobile phase: Linear gradient from 5% Ethanol in heptane to 95% ethanol in heptane in 10 minutes and hold at 100% ethanol for 2 minutes
- Detection wavelength: 254/215nm
- Mass detection: M+1 with APCI mode
- Flow rate: 1.5ml/min
- Sample concentration: ~0.038mg/ml

# Analytical HPLC/MS



UV detection

Mass detection

Analytical chiral column: cellulose-3, 5u, 4.6mmx25cm;  
Flow rate: 1.5ml/min, Solvent: gradient 5% to 95% ethanol in heptane

# Results for Interconversion in Protein Containing Media

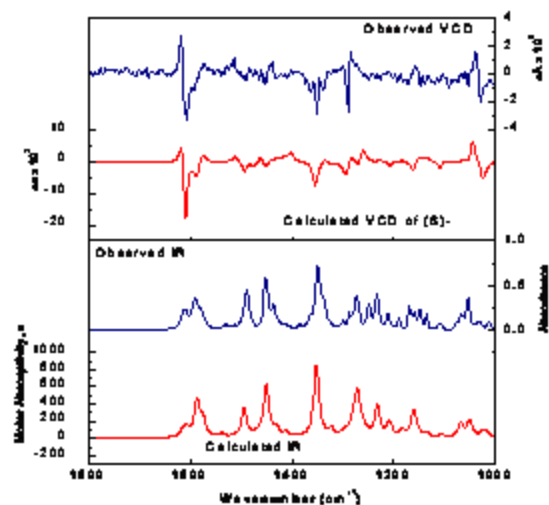
- In all matrices tested, separation of the racemate was unchanged. No racemization was detected for either atropoenantiomer which can be isolated in the presence of proteins at a physiological temperature of 37°C without concern of interconversion

compounds	24 hours (%ee)	48 hours (%ee)
(racemate)	No change (~0% ee)	No change (~0% ee)
Atropoenantiomer-1	No change (~99% ee)	No change (~99% ee)
Atropoenantiomer-2	No change (~99% ee)	No change (~99% ee)

# VCD for Configuration

- VCD measures the differential absorption of a molecule for left circularly polarized infrared (IR) light versus the right during vibrational transitions of the molecule.
- VCD combines both experimental and in silico computations to provide the AC of chiral molecules. When determining the AC of a single enantiomer the VCD will be determined and compared to the VCD simulation of one of the enantiomers.
- If the measured and the calculated VCDs correlate closely, assignment of AC can be made with high confidence to be that of the simulated AC.

IR (lower frame) and VCD (upper frame) spectra observed for atropoenantiomer-2 compared with the calculated spectra for the (*R*) - (=M) configuration.



# Conclusion

As a thermally sensitive class of chiral compounds, the presence of atropisomers is insufficiently considered among the medicinal chemistry community. Commencing from identification and isolation of atropisomers to the support on interconversion, stability and absolute configuration studies, Analytical Chemistry provides valuable guidance aiding the prosecution of molecular targets.

# Acknowledgement

Authors want to thank Mark Noe, Mike Shapiro, Bruce Rogers on the leadership roles and many valuable feedbacks on this project, and to Deane Nason, Wenjian Xu for providing quality material for isolation on tox. studies, and to Dan Virtue, Bob Depianta, Jim Bradow for assistances in the method developments and purifications