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Chromatographic resolution of atropisomers for toxicity and biotransformation studies in pharmaceutical research

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ABSTRACT

Atropisomerism can be a complex concept for those who have not encountered it before. This paper discusses the experiments for identification, isolation, thermal stability, toxicity and biotransformation of various species. The identified atropisomers are a series of rotational hindered biaryl, rotational hindered amide, ring flip, and macrocycles atropisomers identified using supercritical fluid chromatography (SFC) and high performance liquid chromatography (HPLC). These technologies offered the advantage of separating various atropoenantiomers, atropdiastereomers and mixed atropisomers with other forms of stereoisomers in both analytical and preparative scales. With ultra-performance convergence chromatography (UPC^2), the detection of *N*-oxide atropisomer metabolites can be obtained at very low level thus enabling the observation of conversion in human plasma possible. As the resolution of atropisomers are related to the energy barriers on the rotational axis, a calculated computational protocol was developed to predict the formation. A threshold of 10 kcal/mol was established for possible detection of the atropisomers' existence with chromatographic technologies at room temperature or above. The atropisomer with higher energy barrier (>20 kcal/mol) were isolated via preparative chromatography and the isolates studied in vitro and in vivo for evaluation of their stability in human plasma. The detailed analytical method development to analyze the biotransformation of the atropisomers in human plasma are also discussed in this paper.

1. Introduction

Atropisomerism is a stereochemical phenomenon that exist as enantiomeric forms due to restricted rotation around certain single bonds, which behave as chiral axes. The word atropisomers is derived from the Greek "a" which means not and "tropos" which translates as turn [1]. The name was coined by Kuhn in 1933, but atropisomerism was first detected in 6,6′-dinitro-2,2′-diphenic acid by Christie in 1922 [2]. Oki defined atropisomers as conformers that interconvert with a half-life of more than 1000 s at a given temperature [3]. Therefore, even biaryl compounds with a small degree of steric hindrance can resolve into individually isolatable atropisomer if sufficiently cooled. Atropisomers are an important class of compounds because they display axial chirality. The concept of atropisomerism in drug discovery has been properly discussed in several papers [4–6]. The detailed investigations on the rotational barriers in relation to the substituents and substitution pattern of biphenyl congeners were carried out by the research groups of Schurig et al. [7] and König et al. [8]. Like traditional racemic material, atropisomers with a large energy barrier can be isolated by traditional enantioselective chromatography, crystallization, or classic resolution. Chromatographic resolution of chiral molecules offers the advantages of speed, ability to generate both enantiomers for biological tests and higher sample recovery when compared to the other approaches such as asymmetric synthesis or classic resolution of a racemic mixture into individual enantiomers [9]. Enantioselective separation is based on the intermolecular interactions involved in the retention and discrimination processes inside the polysaccharide-based CSPs is an unsolved problem. Several works devoted to this topic allowed to overcome the pioneering three point interaction model through the identification of repulsive interaction, conformational adjustment of the analyte to promote the steric fit into the chiral groove and, more recently, the possibility to use stereoselective halogen bonds as a new tool to drive chiral recognition besides the well-known hydrogen bonding, *p*–*p* and dipole–dipole interactions

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[10]. Atropisomers with sufficient energy barriers can form the insertion complex into the chiral cavities of polysaccharide phases thus leading to chiral recognition. Once isolated, the individual atropoenantiomers will rotate the plane of polarized light in equal but opposite directions and can potentially differ significantly in their pharmacological properties and profiles. They differ from the other chiral compounds in that they can be equilibrated thermally whereas in the other forms of chirality, isomerization is usually only possible chemically [11]. Therefore, the assessment on their thermal stability and the biotransformation in vitro and in vivo human plasma are critical to understand their chemical behaviors. However, biotransformation analysis of atropenantiomers in human plasma is a challenging task as the sample concentration range is typically very low, ~30 μg/mL range, in vitro, and <1 μg/mL range in vivo. The example of the analysis is discussed in this paper.

Four types of atropisomers were developed in various research programs throughout Pfizer drug discovery programs. The first type is the biaryl atropisomers in which literature is generally restricted to biphenyl and to some extent binaphtyl derivatives [12]. Second are rotational hindered amide atropisomers [13]. The third and fourth type are the macrocyclic [14] and ring flip atropisomers [15], respectively. The examples of these compounds are listed in Fig. 1. In several discovery programs pursued at Pfizer, a series of rotationally hindered biphenyl atropisomers have been identified using chromatography in which the rotational barrier around the biphenyl ring creates the atropoenantiomers. In addition to the biphenyl species, ~200 types of biaryl and rotationally hindered amide atropisomer entities have been identified utilizing a combination of chromatography and nuclear magnetic resonance (NMR) technology. For these species, an internal computational protocol is utilized based on rotational energy barrier calculations to predict the atropisomer formation. Based on our ~200 samples, a relationship was observed as chromatographic resolution is "most likely" when energy barrier is >20 kcal/mol; chromatographic resolution is "maybe" when energy barrier is between 10–20 kcal/mol, and chromatographic resolution is "not likely" when energy barrier is <10 kcal/mol. For the macrocycle and ring flip atropisomers species in which scaffolds or multiple bond rotation allow suitable spatial arrangement for separation by SFC or HPLC, however, no computational protocol currently exists internally to predict.

In this paper, we have discussed the resolution of various atropisomers using HPLC and SFC throughout the early stage drug discovery programs. The chromatographic resolution based on the substitutes of the biphenyl rings such as the number, size and position of substituted functional groups are discussed. The thermally stability of the atropisomers are also discussed along with isolation at the preparative scale and subsequent cell viability studies showing the isolated atropenantiomers exhibits different biological effects. Additionally, analytical method development to assess biotransformation of atropisomers in human plasma is discussed.

2. Experimental

2.1. Material

The compounds described in this paper are either commercially available compounds obtained from external suppliers or compounds synthesized at Pfizer Global Research and Development laboratories. Solvents and stationary phases are all commercially available from the various suppliers and described within the text.

2.2. Analytical SFC systems

The analytical SFC systems utilized for the duration of the study are described below, each equipped with solvent and column switching capabilities operating at a system back-pressure of 120 bar, column temperature fixed at 35 °C and a linear gradient from 5 to 60% co-solvent at a total flow rate of 3 mL/min. The initial studies were conducted on the Berger analytical systems from Waters (Pittsburgh, PA, USA), and later on these systems were replaced with Aurora analytical systems from Agilent (Anaheim, CA, USA). The sample is dissolved in methanol. ~10 μL is injected for each run.

SFC System 1: Agilent 1100 and Aurora A5 module (Anaheim, CA, USA).

Column: 250 × 4.6 mm, 5 μm	Solvent:
ChiralPAK AD-H	Methanol
ChiralPAK AS-H	Ethanol
Chiralcel OD-H	Isopropanol
Chiralcel OJ-H	Acetonitrile
ChiralPAK IC	Methanol w/0.2% IPAm[a]
ChiralPAK ID	Ethanol w/0.2% IPAm[a]
	2-propanol w/0.2% IPAm[a]
	Acetonitrile w/0.1% TFA[b]

SFC System 2: Agilent 1100 and Aurora A5 module (Anaheim, CA, USA).

Column: 250 × 4.6 mm, 5 μm	Solvent:
Cellulose-1	Methanol
Cellulose-2	Ethanol
Cellulose-3	Isopropanol
Cellulose-4	Acetonitrile
Amylose-2	Methanol w/0.2% IPAm[a]
ChiralPAK IA	Ethanol w/0.2% IPAm[a]
	2-propanol w/0.2% IPAm[a]
	Acetonitrile w/0.1% TFA[b]

SFC System 3: Waters analytical method station (Pittsburgh, PA, USA).

Column: 250 × 4.6 mm, 5 μm	Solvent:
ChiralPAK IA	9:1 Ethyl Acetate:Methanol[c]
ChiralPAK IB	9:1 Tetrahydrofuran:Methanol[c]
ChiralPAK IC	9:1 Dichloromethane:Methanol[c]
ChiralPAK ID	
ChiralPAK IE	
ChiralPAK IF	

[a] Isopropyl Amine (IPAm), 0.2%v/v.
[b] Trifluoroacetic Acid (TFA), 0.1%v/v.
[c] Either TFA or IPAm at 0.1% v/v or 0.2% v/v.

2.3. Analytical ultra-performance convergence chromatography system (UPC^2)

The analytical UPC^2 system was utilized for the biotransformation studies in addition to the temperature and pressure studies for the low energy barrier atropisomers. The system is supplied from Waters (Milford, MA, USA) and equipped with diode-array UV, evaporative light scatterring (ELSD) and mass spectrometry detectors. The system was set-up with four chiral columns: ChiralPAK-AD-H, ChiralPAK-AS-H, Chiralcel-OD-H, Chiralcel-OJ-H and 4-solvents: methanol, ethanol, acetonitrile, and methanol with 0.1% trifluoroacetic acid. All columns utilized on the system are 4.6 mm × 150 mm with 5 μm particle size. The temperature, pressure, and solvent program are varied dependant on the application purpose, total flow rate of 2.0 mL/min was maintained for all studies. The sample is dissolved in methanol. ~1 μL is injected for each run.

2.4. Analytical normal phase chiral HPLC

The analytical normal phase HPLC system is a hyphenated LC-MS system from Agilent (Anaheim, CA, USA). The six normal phase HPLC columns include: Cellulose-1, Cellulose-2, Cellulose-3, Cellulose-4, Amylose-2 and ChrialPAK-AD-H, 4.6 mm × 250 mm with 5 μm particle size maintained at ambient temperature. Linear