Separation of enantiomers and conformers of Tofisopamon
Using Daicel immobilized polysaccharide-derived chiral columns using the Agilent 1260 Infinity Analytical SFC System

Application Note

Pharmaceuticals

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Abstract

Due to different physiological activities, the chromatographic separation of enantiomers is an important task in drug discovery and development. The determination of the enantiomeric excess (ee) is often done with supercritical fluid chromatography (SFC) in combination with chiral stationary phases. Since finding the right stationary phase that provides separation of the enantiomers is difficult to predict, automated screening of different stationary and mobile phases is advantageous. In this Application Note, the screening of different mobile and stationary phases for the separation of the enantiomers and conformers of Tofisopam is shown using the Agilent 1260 Infinity Analytical SFC and Daicel immobilized polysaccharide-derived chiral columns.
**Introduction**

Tofisopam [1-{3,4-dimethoxyphenyl}-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine] is a member of the 2,3-benzodiazepine compound family. It has been applied as a pharmaceutical in the treatment of anxiety and alcohol withdrawal\(^1\)\(^2\).

Due to its stereogenic center at C(5)-atom, Tofisopam exists as two enantiomers (R(+) and S(–)). Upon dissolution, its diazepine ring system will exist in two boat conformations, leading to two conformers for each enantiomer (Figure 1). The driving force for conformer transition is attributed to the steric repulsion effect between C(4)-methyl and C(5)-ethyl groups.

As a result of the pharmacological interest in Tofisopam\(^3\), it is essential to have an easy and robust method to separate the four isomers in order to individually evaluate their different biological activities, to understand the kinetics and thermodynamics, and to control the quality of the drug.

In this Application Note, we have evaluated the separation of all enantiomers and conformers of Tofisopam with a single, robust, and fast chromatographic method using columns packed with the immobilized polysaccharide-derived chiral stationary phases (CSPs) in combination with the advanced SFC technology of the Agilent 1260 Infinity Analytical SFC System.

**Experimental**

**Chemicals**

The chiral columns CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC, and CHIRALPAK ID used in this study are manufactured by Daicel Chemical Industries, Ltd. They are sized 4.6 × 150 mm id and packed with 5-µm particles of immobilized amylose–or cellulose-derived CSPs.

As the main mobile phase component, supercritical CO\(_2\) (industrial quality 4.8) was used in this study. Different mobile phase modifiers such as methanol (MeOH), 2-propanol (2-PrOH), and acetonitrile (ACN) were screened in different concentrations. All solvents used were HPLC quality. Due to the basic nature of the Tofisopam molecule, diethylamine (DEA) was added to all mobile phases (0.1% v/v).

**Instruments**

All SFC experiments were carried out on an Agilent 1260 Infinity Analytical SFC System. The system contains the A5 fusion module for CO\(_2\) pre- and post-conditioning and a modified Agilent 1260 Infinity Binary LC System for accurate and constant metering of the mobile phase. The system (G4309A) consisted of the following modules:

- SFC Fusion A5 module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity Standard Degasser
- Agilent 1260 Infinity Standard Autosampler
- Agilent 1260 Infinity Diode Array Detector with high pressure SFC flow cell

In addition, an Agilent SFC Method Development kit was used consisting of:

- Two Agilent 1260 Infinity Thermostatted Columns Compartments with built-in valve drives
- Agilent ZORBAX Method Development Valve Kit, 600 bar

**Chromatographic conditions**

Throughout the experimental work, the flow rate was set at 3.0 mL/min, the temperature of the column compartments at 35 °C and the back pressure of CO\(_2\) supercritical fluid at 150 bar.
Results and Discussion

Due to their remarkable enantioselectivity and versatility, CSPs based on polysaccharide derivatives have been widely used for separation of enantiomers or stereoisomers by LC and SFC. Since 2004, the advanced immobilized version of the polysaccharide-based CSPs has been successfully integrated into the toolbox for chiral separation. These new columns have the advantages of universal solvent compatibility, creation of new selectivity profiles, and material robustness. In addition, SFC is considered as an advantageous technique over LC due to its improved diffusion properties, more favorable mass transfer characteristics, and low mobile phase viscosity which result in faster separation. The simultaneous separation of the enantiomers and conformers of Tofisopam has been evaluated in our laboratories with the combination of SFC and the four immobilized chiral columns: CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC and CHIRALPAK ID. Various mobile phase modifiers were examined in a systematic way, including MeOH, 2-PrOH, and ACN.

As shown in Figure 1, R(+)- and S(–)-Tofisopam are the dominating conformers where the ethyl group attached to the C(5)-atom has a quasi-equatorial orientation (Eq.). Depending on the solvent in which the product is dissolved, the conformational equilibriums will move in favor of the formation of R(–)- or S(+) - conformers where the ethyl group attached to the C(5)-atom will change to the axial orientation (Ax.). Our study on conformer transition of Tofisopam in various sample media (MeOH, EtOH, 2-PrOH, Methyl tert-butyl ether/MeOH 90/10 and ACN) indicate that MeOH induces the fastest formation in the highest proportion (up to 26%) of the R(–)- or S(+) - conformers, while ACN leads to the slowest kinetics and in the lowest proportion (up to 16.5% only) of the same molecules. In no case, does the presence of the R(–)- and S(+) - conformers overtake that of the R(+) - and S(–) - enantiomers in the sample mixture. As a consequence, the R(+) - and S(–) - enantiomers (Eq.) are always chromatographically eluted as major peaks and the R(–)- or S(+) - conformers (Ax.) are always eluted as minor peaks. The relative elution order between the R(+) - and S(–) - and between the R(–)- or S(+) - isomers were not determined in our study due to the lack of the reference standards. To facilitate the discussion, we denote here the minor peaks as P(Ax.) and the major peaks as P(Eq.) without assigning the absolute configuration of the molecules in each pair of enantiomer-conformers.

MeOH appears to be the most versatile co-solvent in resolution of Tofisopam isomers. As indicated in Table 1, the best resolution of the four peaks was achieved with CHIRALPAK IA and CHIRALPAK ID. Analysis times can be as short as 3 minutes (Figure 2 (a)) or 5 minutes (Figure 2 (b)), respectively. Interestingly, the apparent elution order is the same on CHIRALPAK IA, CHIRALPAK IC, and CHIRALPAK ID, that is, P(Ax.) - P(Eq.) - P(Ax.) - P(Eq.) Co-elution of two P(Ax.) and one P(Eq.) is observed on CHIRALPAK IB.

<table>
<thead>
<tr>
<th></th>
<th>CHIRALPAK IA</th>
<th>CHIRALPAK IB</th>
<th>CHIRALPAK IC</th>
<th>CHIRALPAK ID</th>
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<td>(t_4)</td>
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<tr>
<td>(R_s(32))</td>
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<tr>
<td>(R_s(24))</td>
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<td>2.04</td>
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</table>

Table 1
Resolution of Tofisopam enantiomers and conformers with MeOH/CO\(_2\)
Co-solvent: 20% (MeOH/DEA 100/0.5 v/v) for CHIRALPAK IA and IC; 10% (MeOH/DEA 100/1.0 v/v) for CHIRALPAK IB; 40% (MeOH/DEA 100/0.25 v/v) for CHIRALPAK ID
The nature of the modifier in SFC can greatly impact the resolution of the four peaks. For instance, the complete resolution on CHIRALPAK IA between the two first eluting peaks is compromised when changing from MeOH (Figure 2 (a)) to 2-PrOH (Figure 2 (e)). In order to achieve full resolution of the first two eluting peaks with this modifier, a gradient run proved to be a good approach (Figure 2. Rs$_{(12)}$ = 1.27 in (e); Rs$_{(12)}$ = 4.18 in (f)). Modifiers also have an effect on elution profile of Tofisopam peaks. This is demonstrated by the separation examples on CHIRALPAK IC and CHIRALPAK ID.

On CHIRALPAK IC, the apparent elution order is $P_{Ax} . P_{Eq} . P_{Ax} . P_{Eq}$ with 20% MeOH (Figure 2 (c)), but becomes $P_{Ax} . P_{Eq} . P_{Eq} . P_{Ax}$ with 50% ACN (Figure 2 (g)). On CHIRALPAK ID, the four species are eluted in the order of $P_{Ax} . P_{Eq} . P_{Ax} . P_{Eq}$ with 40% MeOH (Figure 2 (d)), but changes to $P_{Eq} . P_{Ax} . P_{Ax} . P_{Eq}$, while switching to 40% 2-PrOH (Figure 2 (h)).
The separation of Tofisopam iso-
mers by SFC is perfectly reproduc-
ible, as demonstrated by 120 injec-
tions of Tofisopam over 20 hours
on CHIRALPAK IC under the given
chromatographic conditions (Figure 3).
This can be attributed to the high per-
formance of the Agilent 1260 Infinity
SFC system providing superior CO₂ pre-
and post-conditioning in combination
with precise mobile phase metering.
Such a system performance is depicted
in Figure 4 for the CO₂ back (or outlet)
pressure (a) and the pressure of the
Agilent 1260 Infinity SFC Binary
Pump (b).

**Conclusion**

The Daicel columns packed with immo-
bilized polysaccharide-derived chiral
stationary phases are highly robust for
multiple separation solutions for enan-
tiomers and conformers of Tofisopam.

The complete resolution of Tofisopam
isomers in a single chromatographic
run can be achieved in SFC and HPLC
on the same set of chiral columns7. SFC
is the preferred choice for faster sepa-
rating of Tofisopam. The high instru-
ment performance of the Agilent 1260
Infinity Analytical SFC System renders
the analytical separation of Tofisopam
robust and reproducible.

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**Figure 3**
Reproducibility of the separation on CHIRALPAK IC
Modifier: 30% (MeOH/DEA 100/0.33 v/v), Flow rate: 3 mL/min, Temperature: 35 °C
(a) Retention time; (b) Resolution degree

**Figure 4**
Pressure stability of the Agilent 1260 Infinity Analytical SFC System
Modifier: 30% (MeOH/DEA 100/0.33 v/v), Flow rate: 3 mL/min, Temperature: 35 °C
References


