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Method validation and determination of enantiomers and conformers in tofisopam drug substances and drug products by chiral high-performance liquid chromatography and kinetic and thermodynamic study of the interconversion of the conformers

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Abstract

1-(3,4-Dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine (tofisopam) contains one chiral center, so two enantiomeric forms exist. The ring system of tofisopam possesses two sterically stable boat structures, leading to two distinct conformers for each enantiomer. A method was developed for the separation of these enantiomers and conformers in the drug substances and drug products. Separation was achieved with a separation factor of at least 3.9 for any adjacent peaks. Validation of the method challenged linearity, limit of detection, limit of quantification, specificity, accuracy, repeatability, intermediate precision, robustness, and stability of standard and sample solutions, and all validation results met the acceptance criteria. A study of accuracy at 80%, 100%, and 120% levels gave recoveries of $100 \pm 1\%$. The RSD of six sample injections for repeatability was less than 0.5%. The detection limit of tofisopam enantiomer was as low as 0.12 μg/mL. The kinetics and thermodynamics of the interconversion of tofisopam conformers were also investigated, and the kinetic and equilibrium constants of the interconversion process were determined at 15 °C, 25 °C, and 35 °C.

Keywords: Tofisopam; Enantiomer; Conformer; Chiral separation; Interconversion; Equilibrium constant; Half-life

1. Introduction

Investigation of the separation and characterization of racemic 1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine (tofisopam), an anxiolytic agent, is of intense interest due to the differences in biological activity of the two enantiomers in the living organisms [1]. Pharmacological investigations suggested that each stereoisomer possesses different biological activities not shared by the other, as well as different binding behaviors to human serum albumin (HSA) [2–4]. Tofisopam has a chiral center at the C-5 position. Since there are two stable conformations in the seven-member ring of the chiral benzodiazepine, four distinct conformational species exist, namely *R*-(major)-, *R*-(minor)-, *S*-(major)-, and *S*-(minor)-tofisopam (Fig. 1) [5]. These four conformational species of tofisopam can also be designated as *R*-(+)-, *R*-(−)-, *S*-(−)-, and *S*-(+)-tofisopam, respectively, according to their signs of optical rotation [6–8].

It was found [2] that *R*-(+)- or *S*-(−)-tofisopam with an ethyl group at the quasi-equatorial position was the dominating component of racemic tofisopam in the solid crystal state. The interconversion of the conformers occurred upon dissolution of the dominating conformers in solvents [2]. Understanding the kinetics and thermodynamics of this interconversion is important for further biological and pharmacokinetic studies. To achieve this goal, efficient separation of the four conformational species is a prerequisite.

Fitos et al. used a chiral-AGP (α_1-acid glycoprotein) column to separate the four species, but only three peaks were detected because two of the major components co-eluted [9]. Zsila et al. were able to detect four peaks, but the separation of the

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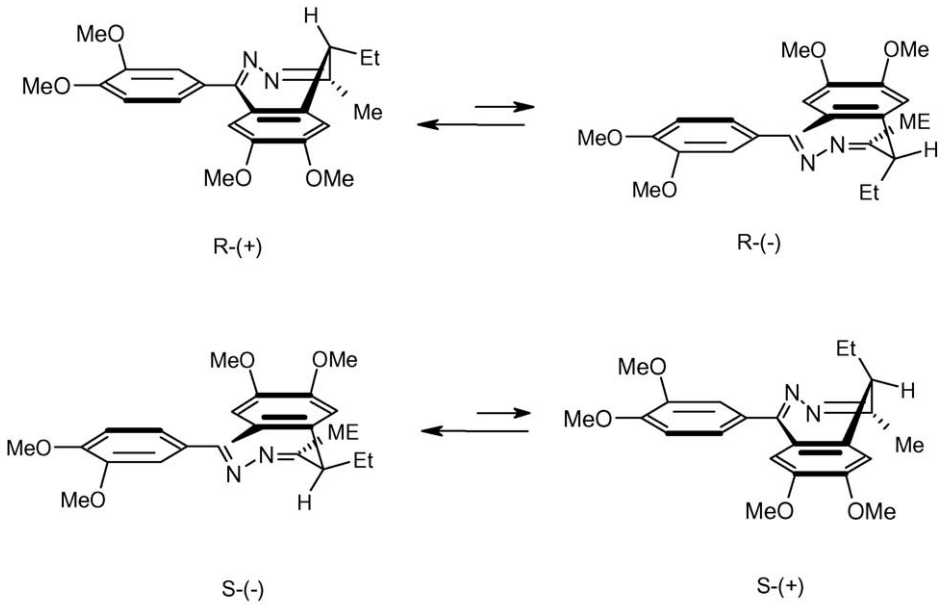

Fig. 1. The structures and conformational equilibria for the enantiomers of tofisopam.

four enantiomer–conformer pairs was not sufficient for accurate quantitation of chiral purity and conformers [10]. Simonyi and Fitos studied the binding selectivity of the enantiomers and conformers of tofisopam to HSA [2]. They prepared a solution of racemic tofisopam and allowed the solution to pass through an HSA-Sepharose column. The chromatograms of racemic tofisopam revealed the binding selectivity for a minor component of *S*-tofisopam (weakly bound) and a major component of *S*-tofisopam (strongly bound). However, the major and minor components of *R*-tofisopam co-eluted, indicating there was no binding selectivity of *R*-tofisopam conformers for HSA. They also performed thermodynamic and kinetic studies of the interconversion of *S*-tofisopam conformers, but no study was reported for the interconversion of *R*-tofisopam conformers since they did not achieve separation due to the non-selectivity of *R*-tofisopam conformers for HSA. Fellefvári et al. investigated the kinetics of the interconversion of tofisopam conformers [11], but the separation of tofisopam enantiomers was not realized. Regis recently used a β-GEM column to try to separate tofisopam enantiomers and conformers, and promising separation was observed [12].

This present work focuses on the investigation of complete separation of the tofisopam enantiomers and conformers in tofisopam drug substances (active pharmaceutical ingredients) and drug products (finished pharmaceutical dosage forms) and accurate measurement of the kinetics and thermodynamics of the interconversion of the conformers.

2. Experimental

2.1. Chemicals

R-Tofisopam and *S*-tofisopam reference standards were provided by Vela Pharmaceuticals (Ewing, NJ, USA). Hexane and ethanol, high-performance liquid chromatography (HPLC) grade, were obtained from EM Science. Triethylamine (TEA; HPLC grade) was purchased from Aldrich. Placebo samples, *R*-tofisopam and *S*-tofisopam capsules were manufactured by The Pharmaceutical Development Service Department, Patheon (Toronto, Canada).

2.2. HPLC analysis

Agilent 1100 HPLC system with data acquisition was utilized for the analysis. The separation was carried out on a β-GEM 1 column (5 μm, 250 mm × 4.6 mm), purchased from Regis Technologies (Morton Grove, USA), with a total analysis time of 35 min. The column was maintained at 25 °C. A mixture of hexane, ethanol, and TEA with the volume ratio of 50:50:0.2 was used as the mobile phase. The flow rate of the mobile phase was 1.0 mL/min, and the sample injection volume was 15 μL. The effluent was monitored by UV (ultraviolet) detection at 310 nm. The sample tray was maintained at 4 °C at all times except for the kinetics and thermodynamic studies during which it was maintained at 15 °C, 25 °C, or 35 °C.

2.3. Kinetic study

The sample solutions (ca. 0.2 mg/mL) for the kinetic studies were prepared by dissolving *R*-tofisopam and/or *S*-tofisopam reference standards in a mixture of hexane and ethanol (50:50, v/v). To measure the kinetic and thermodynamic parameters of the interconversion of the conformers, 15 μL of prepared standards were injected onto the chromatographic column immediately after preparation, and after 0.6 h, 1.2 h, 1.8 h, . . ., and 93.0 h. The interconversion rates, the equilibrium concentrations, and other thermodynamic and kinetic parameters were calculated from the relative concentrations of the two conformers at different times.

3. Results and discussion

3.1. Chromatography

Fitos et al. used a chiral-AGP column to examine resolution of enantiomers and conformers of tofisopam [9]. It was considered that enantioselectivity was due mainly to hydrophobic and hydrogen-bonding interactions. Although *S*-minor and *R*-minor tofisopam conformers could be separated well, *S*-major and *R*-major tofisopam conformers showed identical retention [9]. Zsila et al. used hyphenated HPLC (Chiralcel OJ column-cellulose)-CD techniques to separate and identify tofisopam enantiomers and conformers [10]. The C_5–C_2H_5 group at its pseudo-axial or equatorial position was considered to have affected the strength of interaction between the respective adsorption sites of the cellulose surface and the diazepine ring, resulting in the separation. Complete resolution of the four enantiomer–conformer pairs was not achieved, and so this approach was not suitable for accurate quantitation of each of the four conformers.

Pirkle chiral columns have been widely used for chiral separation, specifically for small molecules with aromatic rings [13]. The chiral recognition mechanism has been examined, and it has been suggested that a combined force of π–π interaction, dipole–dipole interaction, and hydrogen-bonding between the chiral selector and the chiral center contributed to chiral separation [13–15]. Tofisopam is a relatively small molecule with a benzodiazepine and a phenyl aromatic rings and a few functional groups as well. The Pirkle column was felt to be a reasonable selection for separation of the four tofisopam enantiomer–conformer pairs.

In this study, β-GEM 1, a π-acceptor chiral stationary phase prepared by covalently bonding *N*-3,5-dinitrobenzoyl-3-amino-3-phenyl-2-(1,1-dimethylethyl)-propanoate to 5 μm silica through an ester linkage, was used to try to separate the four conformational species of tofisopam. After optimization of chromatographic conditions, a normal phase chiral HPLC method was successfully developed. This method used a Pirkle β-GEM 1 column with a mixture of hexane, ethanol, and TEA (50:50:0.2, v/v/v) as the mobile phase and a UV detector at a wavelength of

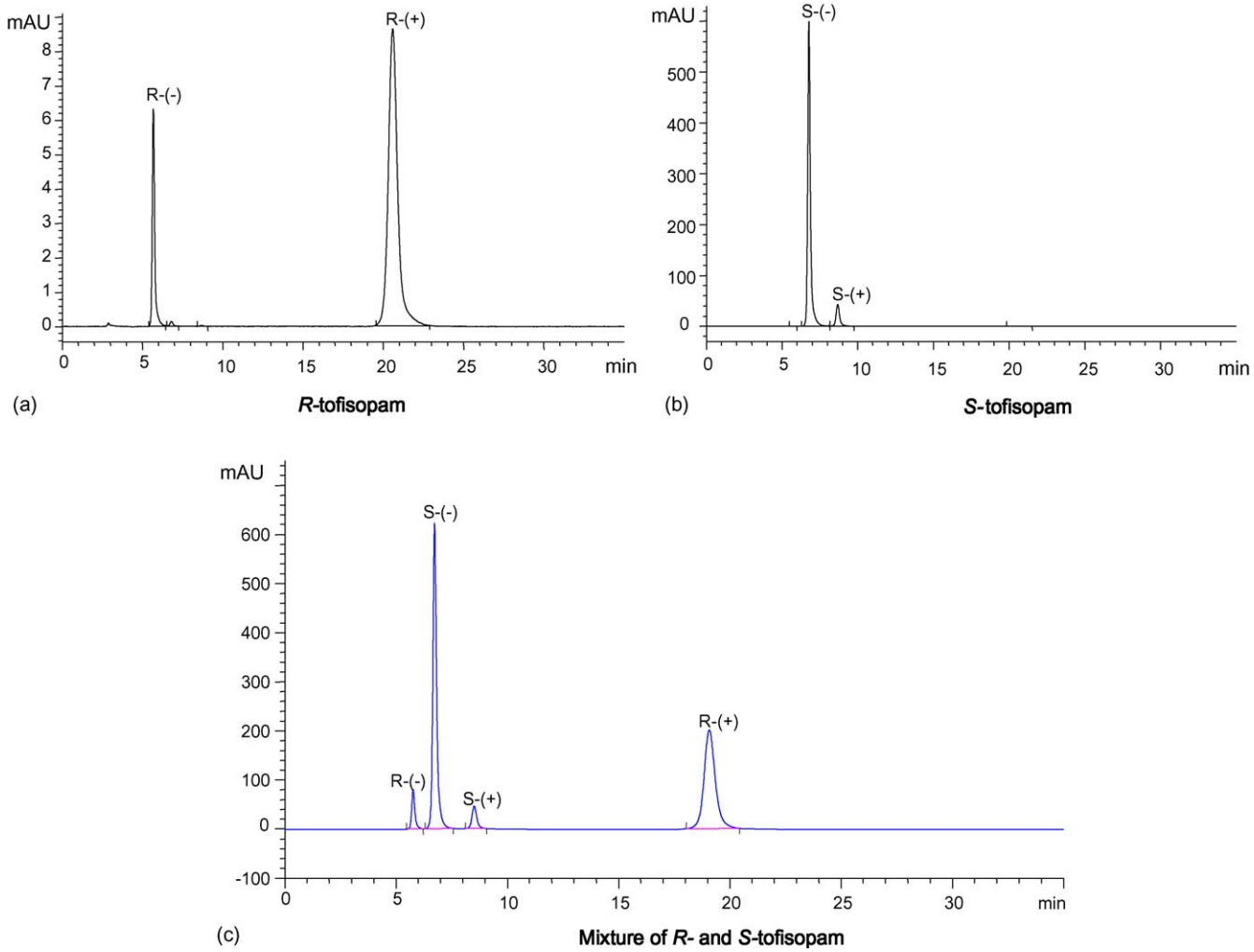

Fig. 2. Typical chromatograms of (a) a standard of *R*-tofisopam (~10 μg/mL), (b) a standard of *S*-tofisopam standards (~0.2 mg/mL), and (c) a mixture of *R*-tofisopam and *S*-tofisopam standards.

Table 1
Summary of method validation for the determination of tofisopam in *R*-tofisopam drug substance

	R-Tofisopam	*S*-Tofisopam
System suitability		
Injection reproducibility	RSD = 0.1%	RSD = 0.4%
USP tailing factor	1.2	1.3
Theoretical plate number	6807	7989
Resolution	>4	>4
Check standard	100.2%	N/A
System drift	100.3–101.7%	N/A
Detection limit	N/A	0.12 μg/mL (0.07% to *R*-tofisopam concentration, S/N = 4.8)
Quantitation limit	N/A	0.39 μg/mL (0.22% to *R*-tofisopam concentration, S/N = 13.0)
Linearity	157.0–235.6 μg/mL, $r = 0.9992$, $b = 102.2$	0.3922–11.76 μg/mL, $r = 0.9994$, $b = -0.3190$
Accuracy	99.2%, 99.3%, 99.3% for 80%, 100%, 120% levels, respectively	97.8%, 98.1%, 101.0% for 4%, 5%, 6% levels, respectively
Repeatability	Mean % *R*-tofisopam: 95.1% (w/w), RSD = 0.5%	Mean % *S*-tofisopam: 2.6% (w/w), RSD = 6.8%
Intermediate precision	Mean % *R*-tofisopam: 95.5% (w/w), RSD = 0.7%, absolute difference between repeatability and intermediate precision results = 0.4%	Mean % *S*-tofisopam: 2.4% (w/w), RSD = 1.7%, absolute difference between repeatability and intermediate precision results = 0.2%
Stability of standard and sample solutions	Stable for up to 7 days	Stable for up to 7 days

310 nm. The method was able to completely separate the four tofisopam enantiomer–conformer pairs with a separation factor of at least 3.9 for any adjacent peaks. Typical chromatograms of *R*-tofisopam, *S*-tofisopam and their mixture standards are illustrated in Fig. 2. The retention times of *R*-tofisopam minor and major peaks were approximately 5.7 min and 19.8 min, respectively, and the retention times of *S*-tofisopam minor and major peaks were approximately 6.7 min and 8.5 min, respectively, with an overall chromatographic run time of 35 min.

Identification of the two enantiomeric peaks was accomplished directly by parallel chromatography of the corresponding *R*-tofisopam and *S*-tofisopam reference standards. The assignment of the conformer peaks turned out to be more complicated due to the interconversion of the conformers. To accurately assign the conformer peaks, two strategies were used. First, according to the reference standards, the minor components present in the solution were either *R*-(−)-tofisopam or *S*-(+)-tofisopam, while *R*-(+)-tofisopam or *S*-(−)-tofisopam were the major components in the solutions when the interconversion equilibrium was reached [2]. Thus the elution order under the experimental conditions was *R*-(−)-tofisopam {*R*-(minor)}, *S*-(−)-tofisopam {*S*-(major)}, *S*-(+)-tofisopam {*S*-(minor)}, and *R*-(+)-tofisopam {*R*-(major)}, assuming their response factors were approximately the same, which was proved to be true in the kinetic study. The identification was also verified by a second approach, that was used in the kinetic study, which will be discussed later.

Table 2
Summary of method validation for the determination of tofisopam in *S*-tofisopam drug substance

	S-Tofisopam	*R*-Tofisopam
System suitability		
Injection reproducibility	RSD = 0.3%	RSD = 0.3%
USP tailing factor	1.4	1.2
Theoretical plate number	6594	8060
Resolution	≥3.9	≥3.9
Check standard	100.4%	N/A
System drift	100.3%	N/A
Detection limit	N/A	0.082 μg/mL (0.04% to *S*-tofisopam concentration, S/N = 3.5)
Quantitation limit	N/A	0.25 μg/mL (0.12% to *S*-tofisopam concentration, S/N = 10.9)
Linearity	169.9–254.9 μg/mL, $r = 0.9996$, $b = -27.44$	0.2666–22.22 μg/mL, $r = 1.000$, $b = 0.02489$
Accuracy	99.8%, 99.6%, 99.9% for 80%, 100%, 120% levels, respectively	91.3%, 100.2%, 100.3% for QL, 5%, 10% levels, respectively
Repeatability	Mean % *S*-tofisopam: 95.7% (w/w), RSD = 0.2%	Mean % *R*-tofisopam: 3.3% (w/w), RSD = 1.2%
Intermediate precision	Mean % *S*-tofisopam: 95.6% (w/w), RSD = 0.2%, absolute difference between repeatability and intermediate precision results = 0.1%	Mean % *R*-tofisopam: 3.4% (w/w), RSD = 1.2%, absolute difference between repeatability and intermediate precision results = 0.1%
Stability of standard and sample solutions	Stable for up to 3 days	Stable for up to 3 days

Table 3
Summary of method validation for the determination of tofisopam in *R*-tofisopam 100 mg capsules

	R-Tofisopam	*S*-Tofisopam
System suitability		
Injection reproducibility	RSD = 0.3%	RSD = 0.3%
USP tailing factor	1.2	1.4
Theoretical plate number	7018	8211
Resolution	>4.0	>4.0
Check standard	99.6%	N/A
System drift	99.6–100.1%	N/A
Detection limit	N/A	0.13 μg/mL (0.07% to *R*-tofisopam concentration, S/N = 3.5)
Quantitation limit	N/A	0.43 μg/mL (0.24% to *R*-tofisopam concentration, S/N = 12.0)
Linearity	146.7–220.0 μg/mL, $r = 0.999$, $b = 80.01$	0.4268–21.34 μg/mL, $r = 1.000$, $b = -4.699$
Accuracy	99.8%, 100.3%, 100.4% for 80%, 100%, 120% levels, respectively	98.9%, 100.8%, 101.8% for 2.5%, 5%, 10% levels, respectively
Repeatability	Mean % *R*-tofisopam: 96.3% (w/w), RSD = 0.9%	Mean % *S*-tofisopam: 2.40% (w/w), RSD = 2.3%
Intermediate precision	Mean % *R*-tofisopam: 95.3% (w/w), RSD = 0.6%, absolute difference between repeatability and intermediate precision results = 1.0%	Mean % *S*-tofisopam: 2.41% (w/w), RSD = 1.4%, absolute difference between repeatability and intermediate precision results = 0.01%
Stability of standard and sample solutions	Stable for up to 4 days	Stable for up to 4 days

Table 4
Summary of the results for the method robustness of determination of *R*-tofisopma and *S*-tofisopam in *R*-tofisopam 100 mg capsules

Enantiomer	Mean % target (%RSD) compared to standard conditions					
	Column temperature (°C)		UV detection length (nm)		Mobile phase composition (hexane:ethanol:TEA, v/v/v)	
	23	27	308	312	49:51:0.2	51:49:0.2
R-Tofisopam	100.6 (0.2)	100.1 (0.1)	100.0 (0.1)	100.0 (0.1)	100.0 (0.1)	99.8 (0.2)
S-Tofisopam	100.1 (0.2)	100.5 (0.2)	100.1 (0.2)	100.0 (0.4)	99.3 (0.7)	100.0 (0.4)

Values in parentheses are in percent.

3.2. Validation

The developed method was validated for its system suitability, linearity, limit of detection, limit of quantification, specificity, accuracy, repeatability, intermediate precision, robustness, and stability of standard and sample solutions. Tables 1–4 summarize the validation results, which demonstrate that the method is suitable for the determination of the potency and enantiomeric purity of *R*-tofisopam and *S*-tofisopam in tofisopam drug substances and drug products.

3.3. Kinetics and thermodynamics of the interconversion of the conformers

In the solid state, *R*-(+)- and/or *S*-(−)-tofisopam are the dominating conformers. The interconversion of the conformers occurs upon dissolution of solid tofisopam in solvents. The rate of the interconversion was monitored by analyzing a freshly prepared standard solution with time (for up to 93.0 h after dissolution). Fig. 3 presents the interconversion profiles of *R*-tofisopam in a mixture of hexane and ethanol (50:50, v/v) at 25 °C. It can be inferred from the time profile that the conversion of *R*-(+)-tofisopam to *R*-(−)-tofisopam occurred upon dissolution of *R*-(+)-tofisopam. With time, the response of *R*-(+)-tofisopam decreased and that of *R*-(−)-tofisopam increased, while the total response of *R*-(+)-tofisopam and *R*-(−)-tofisopam remained constant. This indicates that the response factor of *R*-(+)-tofisopam is identical to that of *R*-(−)-tofisopam because the interconversion ratio is 1:1. Since *R*-(+)-tofisopam and/or *S*-(−)-tofisopam are the dominant components in solid crystal states [2], and the solutions of *R*-tofisopam and/or *S*-tofisopam were injected immediately after sample preparation, the major

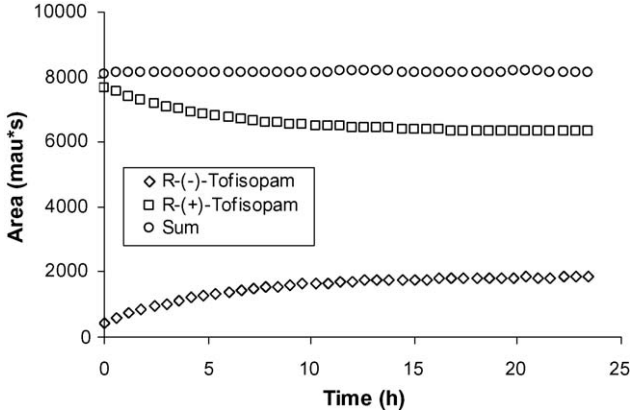

Fig. 3. Time profiles of the interconversion of *R*-(+)-tofisopam and *R*-(−)-tofisopam (25 °C). The total concentration of *R*-tofisopam was 0.2160 mg/mL.

Table 5
Kinetic and thermodynamic constants for the interconversion of *R*-tofisopam conformers at 15 °C, 25 °C, and 35 °C

Temperature (°C)	$k_1 + k_2$ (h^{-1})	K	k_1 (h^{-1})	k_2 (h^{-1})	$t_{1/2}$ (h)	ΔG° (kJ/mol)
15	6.5×10^{-2}	0.28	1.4×10^{-2}	5.1×10^{-2}	10.6	3.3 (3.2[a])
25	0.21 (0.23[a], 0.19[b])	0.29 (0.28[a], 0.27[a])	4.6×10^{-2} (0.05[a])	0.16 (0.18[a])	3.4 (3[a], 3.6[b])	
35	0.49	0.31	0.12	0.37	1.4	

[a] From Ref. [2].
[b] From Ref. [11].

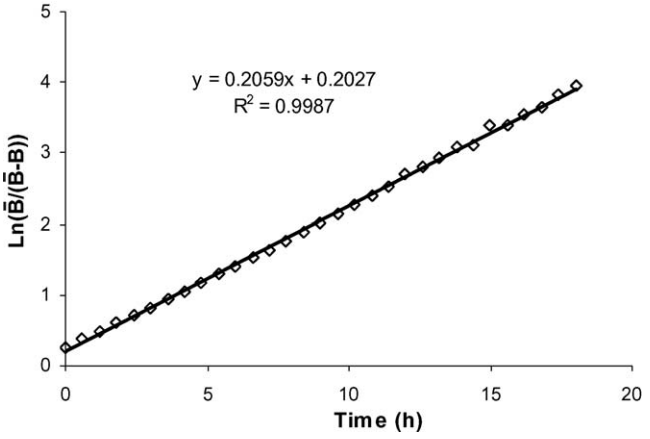

Fig. 4. Kinetic study of the conformational interconversion of *R*-tofisopam (25 °C).

peaks appearing in the corresponding chromatogram would be *R*-(+)-tofisopam or *S*-(−)-tofisopam, and the minor peaks would be *R*-(−)-tofisopam or *S*-(+)-tofisopam. Thus, the peak identification discussed in Section 3.1 was verified by the kinetic study.

The kinetics of the interconversion could be described as follows [10]:

$$A \underset{k_2}{\overset{k_1}{\rightleftarrows}} B \tag{1}$$

where A and B represent the concentrations of the major and the minor conformers, and k_1 and k_2 are the rate constants of interconversion in the two directions. The change of concentrations of the conformers with time (t) is thus as follows [10]:

$$\ln\left(\frac{\bar{B}}{\bar{B} - B}\right) = (k_1 + k_2)(t + t_0) \tag{2}$$

where $\bar{B}$ stands for the equilibrium concentration of the minor conformer and t_0 is the "sample preparation time". Eq. (2) suggests that the left side of the equation should change linearly with time t, and the slope should be the sum of the rate constants k_1 and k_2. Linearity was confirmed experimentally, as demonstrated in Fig. 4. The introduction of parameter t_0 as sample preparation time is due to the non-zero intercept. In practical terms, there is a time gap between sample dissolution and sample analysis, and during this time gap interconversion occurs. In addition, the *R*-tofisopam reference standard may not be 100% pure, and any amount of *R*-(−)-tofisopam will contribute to the non-zero intercept. From k_1 and k_2, the apparent half-life of the interconversion process ($t_{1/2}$) can be estimated as [10]:

$$t_{1/2} = \frac{\ln 2}{k_1 + k_2} \tag{3}$$

When the interconversion equilibrium is reached, the equilibrium constant (K) may be expressed as:

$$K = \frac{k_1}{k_2} = \frac{\bar{B}}{\bar{A}} \tag{4}$$

where $\bar{A}$ denotes the equilibrium concentration of the major conformer.

The values of k_1, k_2, K, $t_{1/2}$, and ΔG° at 15 °C, 25 °C, and 35 °C were determined and these values are summarized in Table 5. All constants at 25 °C agreed well with those reported in the literature. Since the constants from the literature were determined from the interconversion of *S*-tofisopam conformers or that of racemic tofisopam conformers, it can be inferred herein that the thermodynamic equilibrium and kinetic processes of the interconversion of *S*-tofisopam conformers and those of *R*-tofisopam conformers are essentially equivalent to each other. This is reasonable, because *S*-(−)/*R*-(+) and *S*-(+)/*R*-(−) tofisopam are enantiomers and possess the same physicochemical properties, except for optical rotation. The results also suggest that the equilibrium constant increased with the increase of temperature in this solvent system (hexane and ethanol 50:50, v/v). In other words, the amount of conformers B (minor conformers) increased with the increase of temperature, indicating that the reaction of A converting to B is an endothermal reaction ($\Delta H^\circ > 0$). As expected, the time to reach interconversion equilibrium decreased with the elevation of temperature, as demonstrated by the decrease of the half-life of the interconversion from 10.6 h to 1.4 h when the temperature increased from 15 °C to 35 °C.

4. Conclusion

Complete separation of the four conformational species of tofisopam (*R*-(+)-, *R*-(−)-, *S*-(+)-, and *S*-(−)-tofisopam) was realized by the use of (*S*, *S*)-β-GEM 1 chiral column with a mixture of hexane, ethanol, and TEA (50:50:0.2, v/v/v) as the mobile phase. Each chromatographic peak was identified on the basis of parallel analysis of reference standards, reported results in the literature, and a kinetic study of conformer interconversion. The results of method validation demonstrated that the method is suitable for the determination of *R*-tofisopam and *S*-tofisopam in tofisopam drug substances and drug products in terms of system suitability, specificity, limit of detection, limit

of quantification, linearity, repeatability, intermediate precision, robustness, and stability of standard and sample solutions. The established method is also suitable for accurate investigation of the kinetics and thermodynamics of the interconversion of *R*-tofisopam and *S*-tofisopam conformers. The determined kinetic and equilibrium constants of the interconversion will be important for chemical process development and biological activity research.

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