

Research Article

New Discoveries in Enantiomeric Separation of Racemic Tofisopam

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Resolution process of tofisopam has been re-evaluated now based on our new investigations. Originally, it was carried out in the water-chloroform system, where the intermediate salt of high diastereomeric excess was described as *(R)*-TOF-*(R,R)*-DBTA·(H₂O)₃. Opposed to previous assumptions, we have actually found that a different solvate composition, *(R)*-TOF-*(R,R)*-DBTA·CHCl₃, forms with chloroform, in which molecules of CHCl₃ are captured and held with different strengths. Moreover, resolution of TOF with *(R,R)*-DBTA is possible (and favourable) in water-free solvent and solvent mixture. However, presence of chloroform is essential, and thus, chloroform is also a suitable solvent alone. Among the tested solvents, toluene-chloroform mixture results in the highest resolution efficiency, while the highest enantiomeric purity was achieved when acetonitrile was in the system too. Resolution efficiency can be also increased by using the quasi-racemic resolving agent and thermodynamic control. Purification of enantiomeric mixtures was examined, and recrystallization of the diastereomeric salt was found to be the most efficient solution. Instructive behaviour of the complex enantiomer-conformer system of tofisopam is emphasized.

1. Introduction

(*S,R*)-1-(3,4-Dimethoxyphenyl)-5-ethyl-7,8-dimethoxy-4-methyl-5*H*-2,3-benzodiazepine [(*S,R*)-tofisopam, (*S,R*)-TOF] (Scheme 1) is an anxiolytic drug [1, 2], which is the original product of EGIS pharmaceutical company [3].

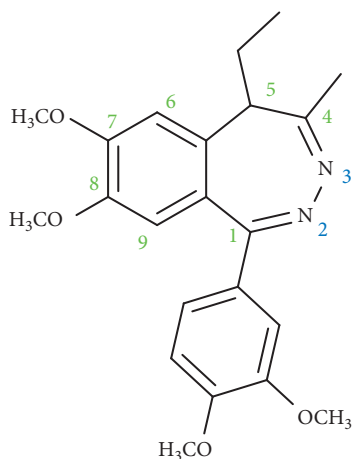
There is no enantiomerically pure form in trade although the effect of tofisopam enantiomers is different. Separation and analysis of enantiomers are described by patents [4, 5] and publications [6, 7]. Since 1986, there was no published discovery about tofisopam resolution and enantiomeric interactions, and thus, we summarized the novel results about it in our study.

1.1. Structure and the Various Biological Effects of Stereoisomers. The structure of the tofisopam molecule is significantly affected by its two subunits: flexible, seven-

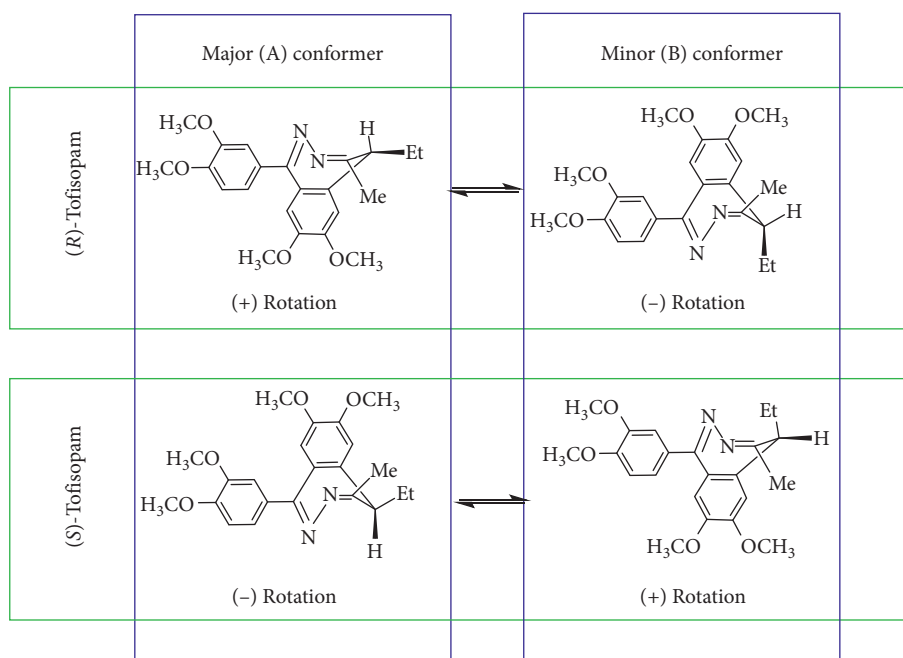
membered ring, and an asymmetric center in it [8]. Based on the ligands configuration for the fifth carbon atom, there are *R* and *S* optical isomers, whose structures are determined by the dynamic equilibrium of two conformers (**A** and **B**). The ethyl group is positioned equatorial in “**A**” or the major conformer, while in “**B**” or the minor conformer, it is positioned axially. Since the former structure is more energetically stable, in the thermodynamic equilibrium established in solution between conformers, the form **A** is the dominant form (**A**:**B** ratio in chloroform solution 85:15) [8].

Due to the helicity of the benzodiazepine moiety, each conformer is a chiral species of relatively high specific optical rotation value [8, 9], so helicity determines the molecular rotation almost exclusively (Scheme 2).

All four advantaged structures have a sedative effect—so they are currently in the racemic form commercially available; however, their efficacy [10, 11] and their metabolism [12] are significantly different.



SCHEME 1: Tofisopam's structure (1-(3,4-dimethoxyphenyl)-5-ethyl-7,8-dimethoxy-4-methyl-5H-2,3-benzodiazepine).



SCHEME 2: The prominent configurations of tofisopam based on the asymmetric center, conformational state, and helicity [10, 11].

Since the turn of the millennium, new research has begun and the so-called dextofisopam ((*R*)-tofisopam) has proved to be suitable for the treatment of gastrointestinal disorders as well [13, 14]. In particular, development of a drug for irritable bowel syndrome was the main target (IBS) [15].

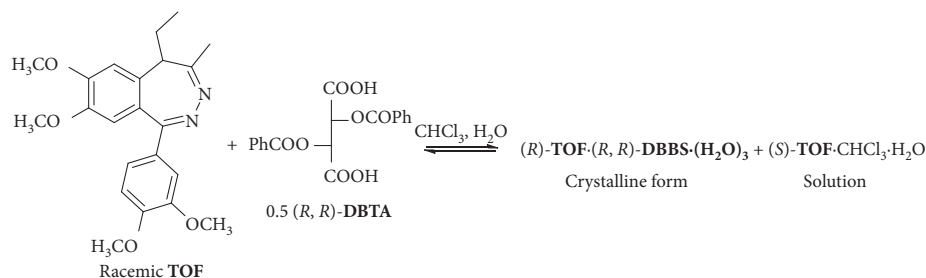
Other effects of stereoisomers are also investigated as inhibitors of PDE4 and PDE10A (enzymes involved in cellular communication) and inhibitors of CYP3A4 (a major metabolizing enzyme) [2, 16, 17].

1.2. Preparation of Enantiomers of Tofisopam. Enantiomers of racemic TOF were separated by (*R,R*)-dibenzoyl-tartaric acid (DBTA) [4]. It was carried out in heterogeneous mixture of water and chloroform (Scheme 2). The solid

phase, separated by two liquid phases, contains (*R*)-TOF·(*R,R*)-DBTA diastereomeric salt. (*S*)-TOF excess was recovered from chloroform solution. It was assumed that salt contains three equivalent water in its crystal, and thus, (*R*)-TOF·(*R,R*)-DBTA·(H_2O)₃ is the composition of the solid phase (Scheme 3).

2. Results and Discussion

2.1. Investigation of Tofisopam Resolution. The first step in enantiomer separation is typically the resolution of the racemic compound. Effects of several factors such as quality of the resolving agent, quantity and quality of solvents, time, and eutectic composition were investigated, respectively. During the analysis, previous assumptions were also re-evaluated [18].

SCHEME 3: Separation of **TOF**'s enantiomers.

2.1.1. Use of a Quasi-Racemic Resolving Agent. We found that use of 0.5 mol (*S,S*)-tartaric acid (**TA**) together with originally used 0.5 mol (*R,R*)-**DBTA** increases separation efficiency of 1 mol racemic tofipam in the water-chloroform system. Application of this quasi-racemic resolving agent [19] results in 26% higher enantiomeric excess and 22% higher resolution efficiency, compared to the original process when (*S,S*)-**TA** is not used (ee: 22.7% $\rightarrow$ 28.5%, *F*: 0.27 $\rightarrow$ 0.33) (Scheme 4). According to NMR measurement, composition of solid diastereomeric salt did not change significantly (**TA** content was only 2 n/n%).

2.1.2. Structure of Diastereomeric Salt. Our investigations confute the previous assumptions since we have observed that diastereomeric salt (obtained from the standard resolution process, Scheme 2) seems to be microcrystalline during the resolution process (Figure 1), and it does not contain water detectably. We found that its composition is (*R*)-**TOF**·(*R,R*)-**DBTA**·*x*CHCl₃, so the salt contains only some chloroform as a solvate or clathrate. During an open air drying, it becomes seemingly more crystalline and loses some CHCl₃. Composition of the crystalline sample will reach a stable composition of (*R*)-**TOF**·(*R,R*)-**DBTA**·*y*CHCl₃, which does not decompose further until it is directly heated (Scheme 5).

According to TG-MS measurement, the air-dried sample loses 5–7% of its mass as chloroform gas until 130°C, and on further heating decomposition starts at around 140°C. At this stage, it loses some more chloroform. Water was not detectable.

Method of TG-FTIR evolved gas analysis (EGA) showed the same results, diastereomeric salt loses its 5–7 wt.% until 125°C, and then CO₂ and PhCOOH-vapor indicate the decomposition. Some more chloroform escapes also in this second step (Figure 2).

According to the NMR measurements, composition of the diastereomeric salt is close to **TOF**:**DBTA** mol ratio of 3:2.

2.1.3. Influence of Chloroform, Water, and Other Solvents. After recognizing that the salt incorporating three water molecules previously described is more likely to be a chloroform solvate and water cannot be detected in it, the question of the need for water was reasonable.

Racemic **TOF** was resolved in small size in chloroform using a half-equivalent (*R,R*)-**DBTA**, and a diastereomeric salt precipitation was observed. The result of the resolution was compared with the separation in the chloroform-water

mixture of the same size. The essential role of chloroform was confirmed. There were no enantiomeric separations between phases without chloroform. Moreover, higher purity was achieved in pure chloroform; however, yield (at the resolution processes, it is calculated on the half of the racemic compound) was higher with water (Scheme 6).

By examining the effect of a third solvent [21], two groups were determined. Methyl ethyl ketone (**MEK**), methyl tetrahydrofuran (**Me-THF**), and acetonitrile (**MeCN**) increased the purity, while toluene (**PhMe**) raised the yield (Figure 3). The yield is calculated for only one of enantiomers (half part of the racemic compound).

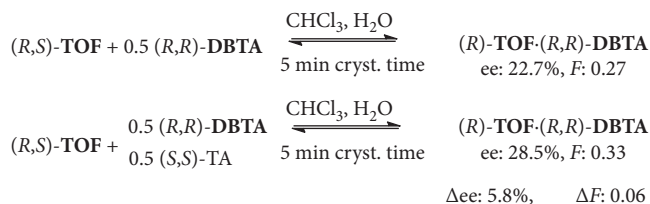
As the essential role of water was rejected, it was substituted by another yield boosting solvent (toluene), and separation efficiency increased significantly (Scheme 7).

2.1.4. Influence of Thermodynamic Control. During the resolution process, enforcement of thermodynamic control increases the enantiomeric excess (ee) of the diastereomer and efficiency of resolution ($F = ee * Y * 10^{-4}$) (0.5 and 24 h crystallization time: ee: 41.5 $\rightarrow$ 67.5%, *F*: 0.37 $\rightarrow$ 0.63). The same effect is observed, when the applied solvent mixture is homogeneous. In this case, less stable minor (*S*)-**TOF** partially dissolves from the initially formed solid phase (Scheme 8).

2.1.5. Influence of the Eutectic Composition. During our previous investigations, we found that the enantiomeric purity of crystallized diastereomers (in a given solvent) correlates with the eutectic composition (ee_{Eu}) of either the racemic compound or the resolving agent. Since **DBTA** forms salt with mixture of 78% major (*R*)-**TOF** and 22% minor (*S*)-**TOF**, only 78% of eutectic composition of **DBTA** or **TOF** can be achieved during the resolution process (thermodynamic control) (Scheme 9).

2.2. Purification of Enantiomeric Mixtures. After resolution, the obtained (*R* > *S*)- and (*S* > *R*)-**TOF** can be purified in form of the diastereomeric salt (by recrystallization and digestion) or enantiomeric mixtures (by re-resolution and recrystallization).

2.2.1. Recover Pure Enantiomers from the Mother Liquor. Recrystallization of the **TOF** enantiomeric mixture from mother liquor was carried out by using methanol and ethyl



SCHEME 4: Results of resolution were improved by applying a quasi-racemic resolving agent.

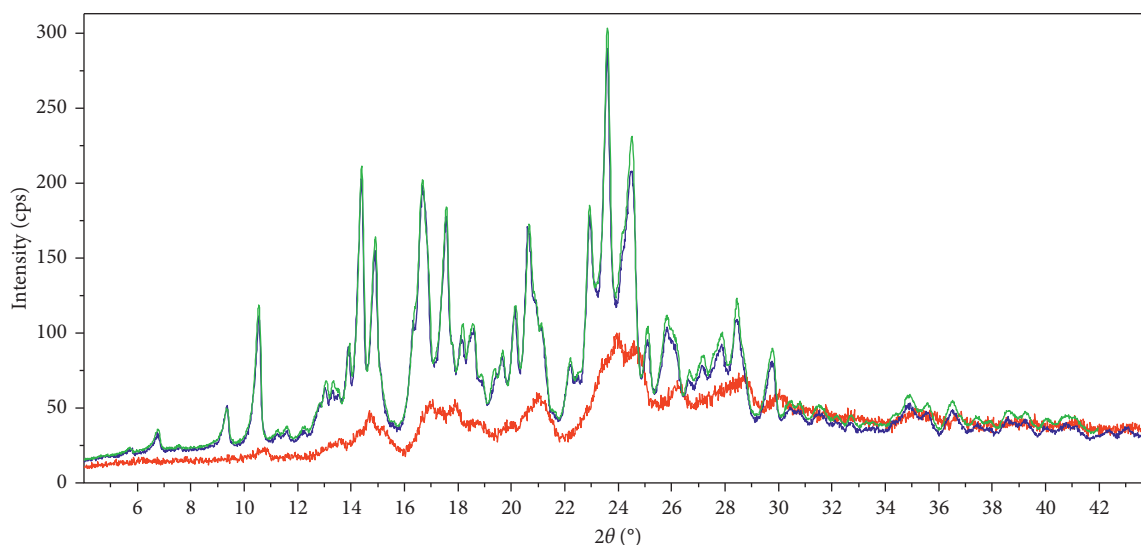


FIGURE 1: Powder XRD profiles of a diastereomeric salt sample, showing the open air drying process starting from its wet state during repeated diffraction measurement series with prolonged measurement time in open air. (—: first run, as short as 3 min; —: immediately repeated run with a longer measurement time of 15 min; —: third run consecutively and taking as long as 40 min.

acetate. Methanol provides higher enantiomeric excess; however, separation efficiency is higher by using ethyl acetate (ee: 52.8 $\rightarrow$ 92.6%, F : 0.37, and 89.7%, F : 0.42, respectively) (Scheme 10).

2.2.2. Re-Resolution of Enantiomeric Mixture. Purification of the same enantiomeric mixture was also carried out by re-resolution (Scheme 11). Separation efficiency is similar to recrystallization although lower purity was achieved. Re-resolution did not work at higher purity (ee₀: 72.4%).

2.2.3. Digeration. The obtained $(R)\text{-TOF}\cdot(R,R)\text{-DBTA}$ salt was purified by digeration with hydrochloric acid (ee: 41.5 $\rightarrow$ 57.8%, F : 0.39) (Scheme 12).

2.2.4. Recrystallization of Diastereomeric Salt. In case of $(R)\text{-TOF}$, the best way to increase purity is recrystallizing the diastereomeric mixtures (ee: 41.5 $\rightarrow$ 81.2%, F : 0.61). In this case, mixture of chloroform, toluene, and water was used as solvent (Scheme 13).

2.2.5. Behaviour of Enantiomers. It is observed that the rate of minor conformer decreases as enantiomeric excess is growing. In the solution of the **TOF** enantiomeric mixture, four conformers are present; however, conformers in diastereomeric relationship are less soluble than conformers in the enantiomeric relationship. This way, enantiomeric mixtures (crystallized from saturated solution during kinetic control) contain less minor conformers as purity increases (Table 1).

Pure enantiomers and racemic compounds transform into different products in their chemical reactions [22]. In case of enantiomers, there is at most one by-product, but racemic mixtures often lead to more ones. The main reason of this difference is that racemic compounds have both homo- and heterochiral interactions, while only homochiral interactions affect in case of enantiomers.

Based on our earlier observations, enantiomers help each other to achieve conformational equilibrium, but the single enantiomer does not have such kind of help. However, it can also achieve the conformation equilibrium, but the single enantiomer does not have such kind of help. Albeit slowly, but here too, the formation of balance is observed because the structure remembers what happens when both enantiomers were present. So, the single enantiomer carries the code of its structure and validates it in its reactions (Scheme 14).

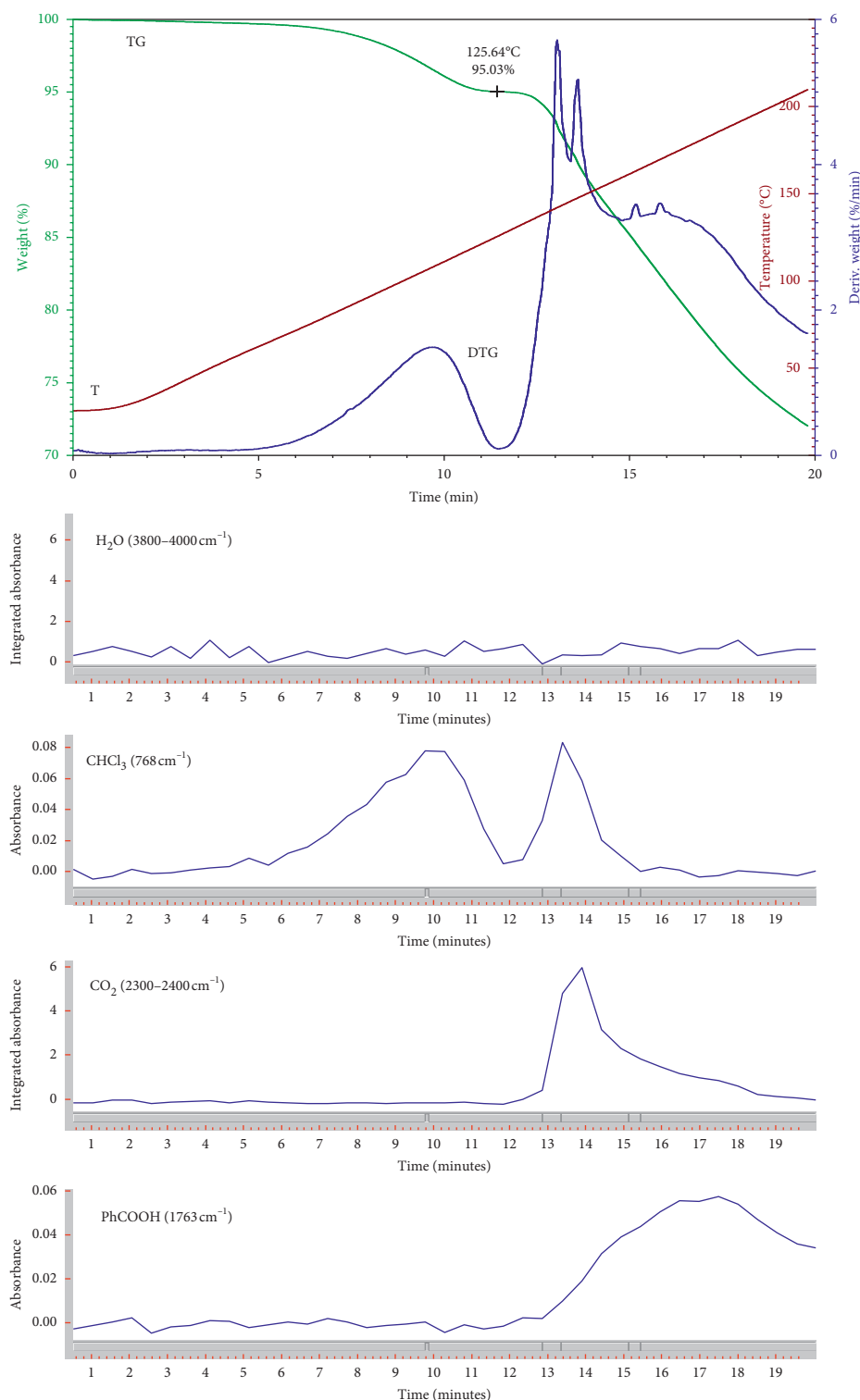
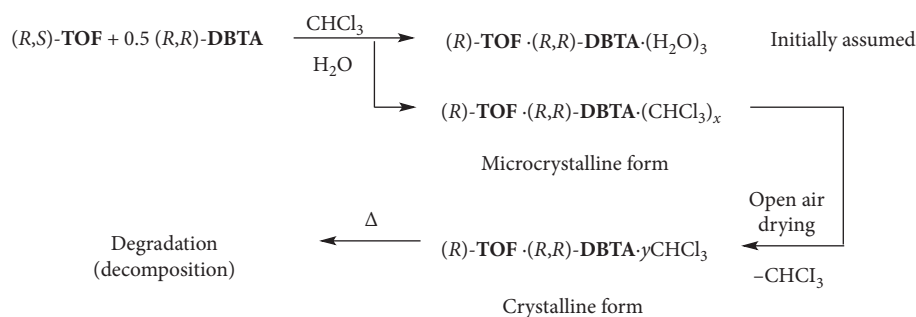


FIGURE 2: Results of a TG-FTIR run of the diastereomeric salt during dynamic heating.

2.3. Material Balance of TOF Enantiomer Separation Process. Finally, material balance of the separation process of racemic **TOF** was made. First, the resolution step was carried out by (*R,R*)-**DBTA** in CH_3CN -toluene- CHCl_3 mixture with 1 week crystallization time. (*R*)-**TOF** (ee: 68.7%, Y: 79.1%, *F*: 0.653) was recovered from the solid diastereomeric salt, while (*S*)-**TOF** (ee: 49.5%, Y: 105%, *F*: 0.520) was recovered

from mother liquor. These enantiomeric mixtures were recrystallized from ethyl acetate, and after a night, we gained (*R*)-**TOF** (ee: 79.1%, Y: 62%, *F*: 0.49) and (*S*)-**TOF** (ee: 89.7%, Y: 44%, *F*: 0.395). Beside them, 40% of original racemic **TOF** was recovered. Taking this into account, yields of (*R*)- and (*S*)-**TOF** are almost 100% (*F*: 0.81) and 73% (*F*: 0.65), respectively (Scheme 15).



SCHEME 5: Composition of the solid phase.

$(R,S)\text{-TOF} + 0.5 (R,R)\text{-DBTA} \xrightleftharpoons{0.5\text{h}} (R)\text{-TOF} \cdot (R,R)\text{-DBTA}$		$(R,S)\text{-TOF} + 0.5 (R,R)\text{-DBTA} \xrightleftharpoons{0.5\text{h}} (R)\text{-TOF} \cdot (R,R)\text{-DBTA}$		
Resolution in pure CHCl_3 solvent		Resolution in 1:1 $\text{H}_2\text{O}-\text{CHCl}_3$ mixture	Resolution in pure CHCl_3 solvent	Resolution in 1:1 $\text{H}_2\text{O}-\text{CHCl}_3$ mixture
$Y = 70\%$	<	$Y = 90\%$	$Y = 70\%$	$Y = 90\%$
$ee = 53.2\%$	>	$ee = 41.5\%$	$ee = 53.2\%$	$ee = 41.5\%$
$F = 0.371$	$\approx$	$F = 0.374$	$F = 0.371$	$F = 0.374$
				Resolution in 1:1 toluene- CHCl_3 mixture
				$Y = 109\%$
				$ee = 48.6\%$
				$F = 0.530$

SCHEME 6: Effect of water on resolution efficiency ($F = Y/100 * ee/100$) [21].

SCHEME 7: Effect of water-toluene substitution on resolution efficiency.

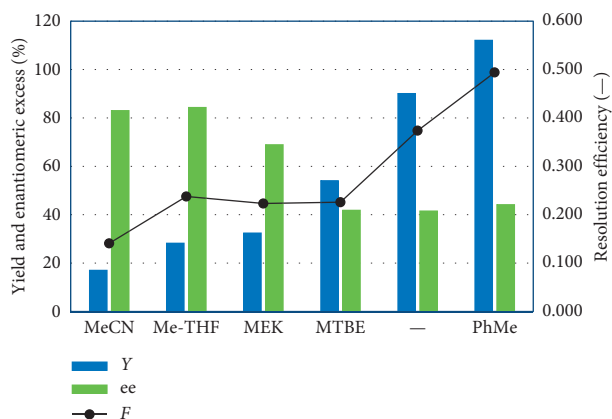
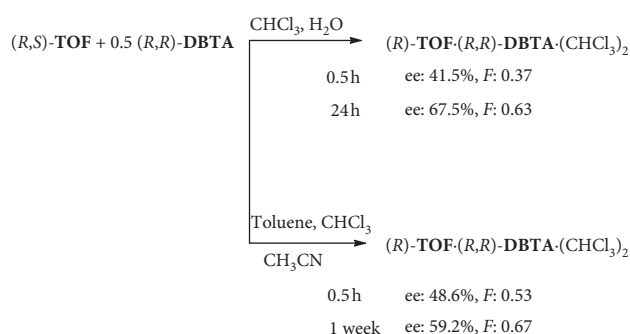


FIGURE 3: Effect of the third solvent on resolution results.

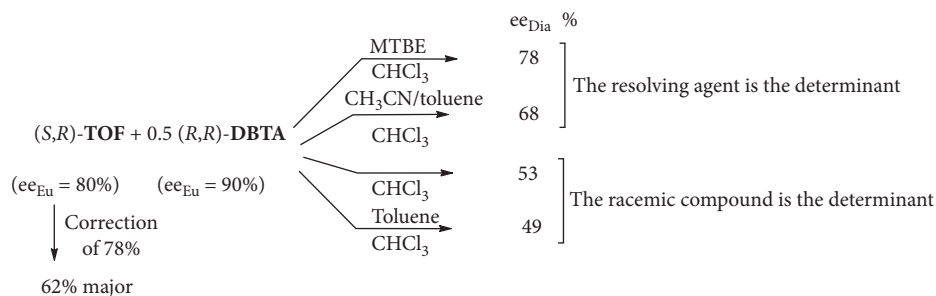
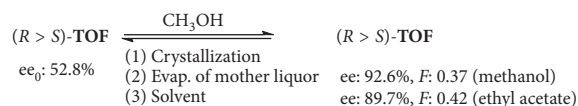


SCHEME 8: The thermodynamic control is more favourable. Minor conformer partially dissolves, while the stable form accumulates (see Section 2.2.5).

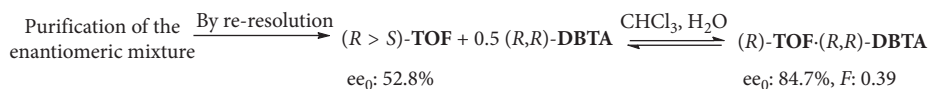
3. Conclusions

Herein, we have investigated the resolution and enantiomer separation of tofisopam and re-evaluated the previous methods and results. Composition of solid diastereomeric salt, obtained from the standard resolution method, was analyzed deeply, and the useful information was applied in development of the resolution process. Essential role of water was rejected since it was not detected during thermoanalytical measurements, and resolution efficiency increased by substituting it with toluene. Crystallization of the diastereomeric salt requires chloroform, and the salt captures and holds it with two different strengths. We found that presence of (S,S)-TA

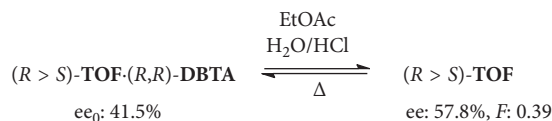
increases the separation efficiency as it forms a quasi-racemic resolving agent with (R,R)-DBTA. Acetonitrile increased the purity in resolution, especially when thermodynamic control could prevail. The enantiomeric excess in diastereomeric salt was influenced by either eutectic composition of the resolving agent (MTBE- CHCl_3 and CH_3CN -toluol- CHCl_3) or racemic compound (CHCl_3 and toluol- CHCl_3). Further purification was carried out by recrystallization, re-resolution of enantiomeric mixtures, and digestion of the diastereomeric salt. The best process was recrystallization of the diastereomeric salt in the optimized solvent mixture. It was observed that the rate of minor conformer decreases as enantiomeric excess is growing. Racemic tofisopam

SCHEME 9: The effect of the racemic compound or resolving agent (ee_{Eu}) is solvent dependent.

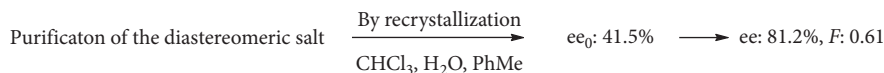
SCHEME 10: Purification of the enantiomeric mixture by evaporation of mother liquor and recrystallization of enantiomeric mixture.



SCHEME 11: Purification of the enantiomeric mixture by re-resolution.



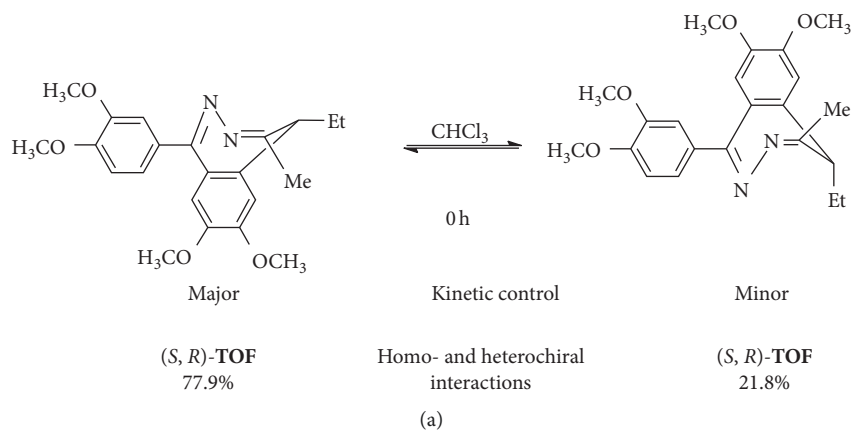
SCHEME 12: Diastereomeric salt can be purified by digeneration with hydrochloric acid.



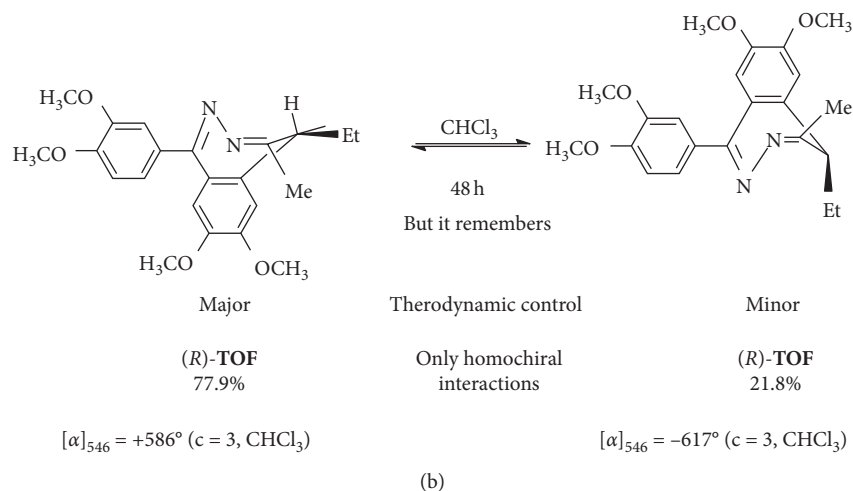
SCHEME 13: Purification of the diastereomeric salt by recrystallization.

TABLE 1: Decreasing rate of minor conformer in the solid phase.

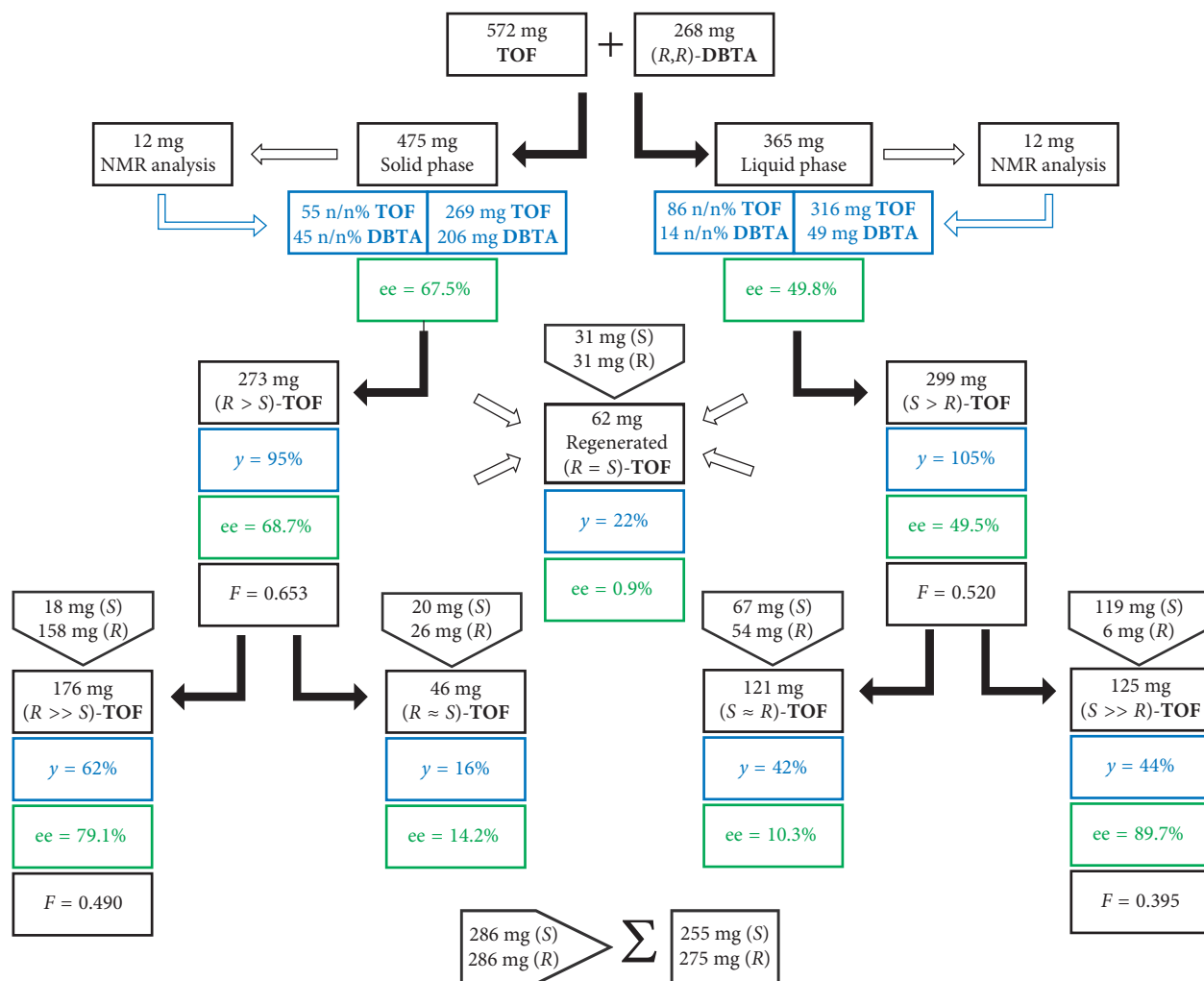
Purity of the solid phase ($ee\%$)	Rate of minor conformer (%)
52.4	33.7
54.9	26.6
84.2	9.4
90.8	5.9



SCHEME 14: Continued.



SCHEME 14: (a) Conformers of TOF; (b) enantiomers remember the original structure and the ratio of conformers in the racemic compound.



SCHEME 15: Material balance of the resolution of racemic TOF by (R,R) -DBTA.

reaches the 78 / 22 major/minor conformer ratio instantly, while single enantiomers need longer time. This can only be explained by supposing that the structure of the

enantiomer is the code of the stabilization of the conformer ratio. The single enantiomer is remembered, and its memory is secured by its structure.

Finally, a new resolution method was applied to separate enantiomers, and we could reach high resolution efficiency by regenerating the racemic excess.

4. Experimental

Chemicals were the products of Aldrich (Steinheim, Germany). Optical rotation data were measured with a PerkinElmer 241 automatic polarimeter.

4.1. Determination of Enantiomeric Excess by High-Performance Liquid Chromatography. Enantiomeric excess (ee) was measured by the reversed-phase high-performance liquid chromatography (HPLC) method. Phenomenex, lux-cellulose-3 chiral column, was used with polar eluent: 70% 20 mM NH_4OAc water buffer and 30% MeCN. Applied volumetric flow was 0.5 ml/min. As a result, three different peaks appeared on the chromatogram: mixture of two minor conformers, major (R)-TOF and major (S)-TOF, respectively. Purity was determined based on the last ones.

4.2. Separation Process of Tofisopam Enantiomers. Firstly, 572 mg (1.5 mmol) racemic TOF (Egis pharmaceuticals plc.) was dissolved in 0.75 ml CHCl_3 and then 0.75 ml MeCN was added. Secondly, 268 mg (0.75 mmol) (R,R)-DBTA (Fluka) was dissolved in 1.5 ml warm chloroform and then it was added to the previous solution. This new yellow homogeneous mixture was diluted by 0.75 ml PhMe and stirred (1000 rpm) at RT. After a week, the yellow solid phase was filtered and washed twice by 1 ml CHCl_3 . Crystals were dried under vacuum. Enantiomeric excess was determined by the HPLC method, and diastereomeric composition was calculated based on NMR measurements.

Mother liquor was concentrated by evaporation, and then the TOF base was recovered the same way as in case of diastereomeric salts. Solids were suspended in 1 ml water, and then 1 ml NH_4OH solution (25%) was added and stirred for 30 minutes. TOF base appeared as yellow oil on the top and was extracted by 1 ml CHCl_3 . Extraction method was repeated twice. Finally, chloroform was evaporated from the solution, and TOF was collected in the form of white powder. Its purity was measured by HPLC.

(R>S)-TOF and (S>R)-TOF were purified by recrystallization from EtOAc. Produced TOF enantiomeric mixture was dissolved in 3 ml EtOAc, cooled down, and kept under 5°C for 12 hours. Finally, the white crystals were filtered and dried, while mother liquors were evaporated. Purity of all the products was measured by HPLC.

Data Availability

The conclusions reached by the authors were based on the measurement data described and reported in the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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