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Configurational stability of 9-hydroxyrisperidone. Kinetics and mechanism of racemization

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ABSTRACT

The configurational stability of 9-hydroxyrisperidone, an atypical antipsychotic, was studied under acidic, basic and physiological conditions. The analysis of 9-hydroxyrisperidone was performed using a recently validated chiral capillary electrophoretic method developed using a dual cyclodextrin mode (hydroxypropylated-β-CD and sulfated-α-CD). The kinetic parameters (rate constants, half-lives, and apparent free energy barriers) of the racemization were calculated through a mathematical model of the first-order reaction. The influences of the pH, the temperature, the nature and the concentration of the buffer, and the presence of an organic co-solvent were investigated. The fastest racemizations were observed under acidic conditions with high phosphate buffer concentrations and high temperatures. Under these conditions, the cyclodextrins (β-CD, methyl- β-CD, or hydroxypropylated-β-CD) added to both enantiomers in various molar ratios were not able to retard the racemization. Finally, the mechanism of racemization was investigated using nuclear magnetic resonance (NMR) and the proton–deuterium exchange of the proton H_9 borne by the chiral carbon has proven the presence of an imine–enamine tautomerism.

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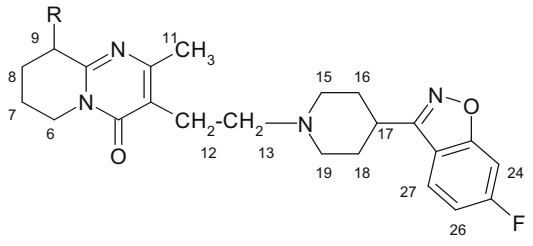
1. Introduction

It is well established that enantiomers often differ in pharmacological activity, toxicity, and pharmacokinetic characteristics and nowadays, about 25% of chiral drugs are administered as pure enantiomers.[1] The study of their configurational stability is then an essential step in the discovery stage of new chiral compounds to make sure that the enantiomers remain configurationally stable under physiological conditions, during the manufacturing process and its shelf-life. It is also essential to study their stability under many other conditions they could meet during further studies, as for example in solubilization solvents or analytical conditions. In this aim and in a continuation of our previous work dealing with the chiral stability of compounds of pharmaceutical interest, the chiral stability of 9-hydroxyrisperidone (9-OHRisp) was investigated.[2]

Risperidone (Risp) (Risperdal®, Johnson & Johnson—Issy-les-Moulineaux, France) is a benzisoxazole atypical antipsychotic agent approved for the treatment of psychosis (including schizophrenia) and also some forms of bipolar disorder, psychotic depression, and Tourette syndrome.[3–6] It is a selective monoaminergic antagonist with a strong affinity for serotonin type 2 (5-HT2) receptors and a slightly weaker affinity for dopamine type 2 (D2) receptors.[7] Risp is metabolized in the liver by cytochrome P450 to produce mainly the active chiral 9-hydroxyrisperidone (Fig. 1)



risperidone : R = H

9-hydroxyrisperidone : R = OH

Figure 1. Chemical structure and assignments of the hydrogen atoms of risperidone and 9-hydroxyrisperidone.

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which shows similar pharmacological activity to that of its parent compound.[8] It has received marketing authorization in the European Union and USA in 2007 under the International Non-proprietary Name (INN) paliperidone (Invega®, Johnson & Johnson). Paliperidone has been developed as a racemate, since both enantiomers have comparable pharmacological activity. However, plasma protein binding of the enantiomers exhibited species-dependent stereoselectivity; the dextrorotatory enantiomer is slightly more abundant in plasma than the levorotatory enantiomer.[9] To the best of our knowledge, no study of the enantiomeric stability of 9-OHRisp has ever been reported in the literature. The product monograph only relates the presence of interconversion in aqueous solutions under acidic and alkaline conditions and no data were given.[9]

Herein, the kinetics of racemization were established under various conditions by studying the influence of the pH, the temperature, the nature and the concentration of the buffer, and the presence of organic co-solvent. The influence of cyclodextrins (CDs) was also investigated since these are pharmaceutical excipients able to retard the racemization rate.[10–12] Lastly, the mechanism of racemization was investigated using nuclear magnetic resonance (NMR).

2. Results and discussion

Previously, the preparation of pure enantiomers of 9-OHRisp by high performance liquid chromatography (HPLC) was performed using the polysaccharide Chiralcel OJ chiral stationary phase and a ternary mobile phase (*n*-hexane/ethanol/methanol, 50/35/15 (v/v/v)).[13] Moreover, three analytical methods of enantioseparation of 9-OHRisp were developed and validated using HPLC or capillary electrophoresis (CE). Since the CE method is developed in aqueous media, it was then chosen to achieve the present study. The optimization of the CE method was based on a central composite design and the response surface methodology and resulted in a baseline enantioresolution in a short analysis time of about 13 min using a dual cyclodextrin mode (hydroxypropylated-β-CD and Sulfated-α-CD).[13,14] It is worth mentioning that previous analysis of the isolated enantiomers using this method has shown that for (+)-9-OHRisp the potential presence of an enantiomeric impurity was inferior to the calculated limit of detection (0.1%). Thus, possible racemization during the electrophoretic analysis (13 min) can be neglected, this is in accordance with our evaluated half-life time (vide infra).

2.1. Kinetics of racemization

The enantiomeric excesses (ee %) were calculated from the concentration of each enantiomer according to their respective UV signal using the calibration plots of the previous validated CE method.[14]

$$\text{ee } \% = \frac{[\text{R}] - [\text{S}]}{[\text{R}] + [\text{S}]} \times 100 \quad \text{if R is the major enantiomer} \tag{1}$$

For a first-order reaction, the linear ln ee versus time plot permits us to determine the apparent constant rate (k_{rac} = −slope) and half-life times ($t_{1/2\,rac} = \ln 2/k_{rac}$), defined as the time required for an enantiopure solution to reach 50% ee (Eq. 2).[15]

$$\ln \text{ee} == -k_{rac}t \tag{2}$$

The apparent free energy barrier ($\Delta G^{\neq}$) is calculated according to the Eyring equation (Eq. 3):

$$\Delta G^{\#} = -RT \ln\left(\frac{k \cdot h}{k_b \cdot T}\right) \tag{3}$$

The major part of the kinetic study of the racemization was achieved for the (−)-9-OHRisp enantiomer since the results obtained are considered similar for both enantiomers. In chiral media, in the presence of CDs—the configurational stability was obviously investigated for both enantiomers.

2.1.1. Influence of the pH and the buffer concentration

Since interconversion of enantiomers had been reported under acidic or basic conditions, the racemization was first studied for various pH: four acidic pH (2.5, 3.5, 4.0, and 4.5), the physiological pH 7.4, and three basic pH (9.0, 11.0, and 12.0).

At acidic pH 2.5, the racemization studies of the (−)-9-OHRisp were performed in phosphate buffers (25, 50, 100, and 125 mM)

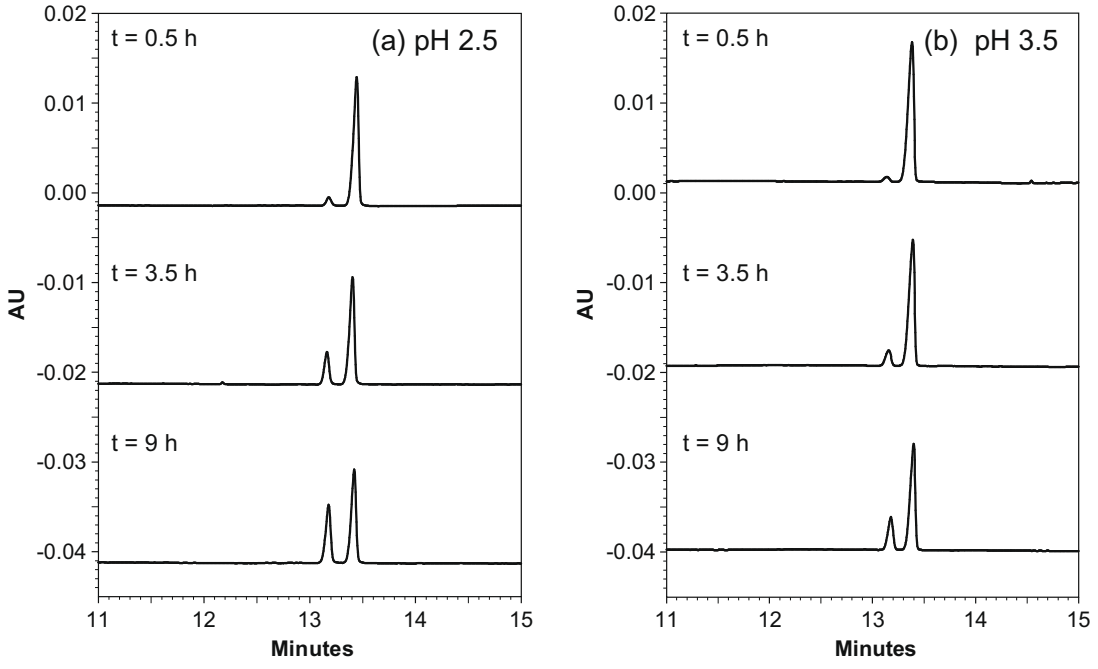

Figure 2. Stacking electropherograms of (−)-9-OHRisp after 0.5, 3.5, and 9 h in a 100 mM phosphate buffer at 37 °C: (a) pH 2.5, (b) pH 3.5.

Table 1
Kinetic parameters (k_{rac} and $t_{1/2\,rac}$) and free energy barriers ($\Delta G^{\neq}$) for the racemization of (−)-9-OHRisp in phosphate buffers pH 2.5 at 37 °C (I = 0.1 M): influence of the phosphate buffer concentration

Phosphate buffer concentration (mM)	k_{rac} (s^{-1})	$t_{1/2\,rac}$ (h)	$\Delta G^{\neq}$ (kJ mol^{-1})
25	1.04×10^{-5}	18.5	105.6
50	1.99×10^{-5}	9.7	103.9
75	3.26×10^{-5}	5.9	102.7
100	4.47×10^{-5}	4.3	101.8
125	5.72×10^{-5}	3.4	101.2

Mean values calculated from three experiments.

Table 2
Kinetic parameters (k_{rac} and $t_{1/2\,rac}$) and free energy barriers ($\Delta G^{\neq}$) for the racemization of (−)-9-OHRisp in a 100 mM phosphate buffer at 37 °C: influence of the pH

pH	k_{rac} (s^{-1})	$t_{1/2\,rac}$ (h)	$\Delta G^{\neq}$ (kJ mol^{-1})
2.5	4.47×10^{-5}	4.3	101.8
3.5	1.85×10^{-5}	10.4	104.1
4.0	1.18×10^{-5}	16.3	105.3
4.5	1.14×10^{-5}	16.8	105.4

Mean values calculated from three experiments.

at 37 °C. In these buffers, convenient amounts of sodium chloride NaCl were added to maintain the ionic strength constant (I = 0.1 M). As illustrated in Figure 2a for the 100 mM phosphate buffer, the racemization phenomenon is relatively fast. The kinetic parameters obtained through the ln ee versus time plots are shown in Table 1 and show the large influence of the phosphate buffer concentration on the racemization, since half-lives vary from 18.5 h to 3.4 h for phosphate buffer concentrations varying from 25 to 125 mM, respectively. The first-order rate constants (in s^{-1}) of racemization appear linearly dependent on phosphate concentrations (in mM), as shown by the linear equation obtained (Eq. 4) (Fig. 3):

$$k_{rac} = 4.70 \times 10^{-7}\ [\text{phosphate}] - 2.30 \times 10^{-6} \quad (4)$$

It is worth mentioning that the influence of the phosphate buffer concentration was investigated with or without addition of NaCl in the phosphate buffers. For example, the $t_{1/2\,rac}$ determined for 100 mM phosphate buffers with I = 0.07 and I = 0.10 are 4.2 h and 4.3 h, respectively, which indicate that the ionic strength of the phosphate buffers has no influence on the racemization phenomenon.

In acidic media, the influence of the pH on the racemization was investigated for 100 mM phosphate buffers. Figure 2b illustrates the racemization of the (−)-9-OHRisp at pH 3.5. The results obtained for pH 2.5, 3.5, 4.0, and 4.5 are enclosed in Table 2 and report the large influence of this parameter on the kinetics of racemization.

For pharmaceutical purposes, the configurational stability of (−)-9-OHRisp was investigated at physiological pH 7.4 and 37 °C. After 100 h, no racemization was observed whatever the nature of the buffer is (100 mM phosphate buffer and 25 mM Tris buffer).

At basic pH, the racemization studies of the (−)-9-OHRisp were first performed in 100 mM borate buffers pH 9.0 or 11.0 at 37 °C. After 100 h, whereas no racemization was observed at pH 9.0, a minor amount of racemization occurred at pH 11.0 since 2.1% of (+)-9-OHRisp were detected. This last result allows to estimate the $t_{1/2\,rac}$ at pH 11.0 of about 3×10^3 h. Last, strong basic conditions pH 12.0 (10^{-2} M sodium hydroxide solution) at 50 °C have provided higher configurational instability with $t_{1/2\,rac}$ equal to 32.4 h.

2.1.2. Influence of the temperature

Since acidic conditions favor configurational instability of the 9-OHRisp, the study was more detailed for pH 2.5 and, to a lesser extent, for pH 3.5. Then, the influence of the temperature was investigated for both pH by determining the kinetic parameter of the racemization process at 10, 25, 30, 37, and 45 °C in the 100 mM phosphate buffer. The results obtained are shown in Table 3 and show the large influence of the temperature on the rate constants. In accordance with the Eyring equation (Eq. 5), the enthalpic and entropic terms can be determined by plotting $\ln(k/T)$ versus $1/T$ (Fig. 4).

$$\ln\left(\frac{k}{T}\right) = \frac{\Delta H}{RT} + \ln\left(\frac{k_b}{h}\right) + \frac{\Delta S}{R} \quad (5)$$

The obtained ΔH and ΔS are, for pH 2.5, 83.4 kJ mol^{-1} and −67.6 J mol^{-1} K^{-1} and, for pH 3.5, 82.2 kJ mol^{-1} and −62.6 J mol^{-1} K^{-1}, respectively. However, due to the relative standard error obtained for these thermodynamic parameters (RSD around 5–8% for three experiments), the enthalpic and entropic

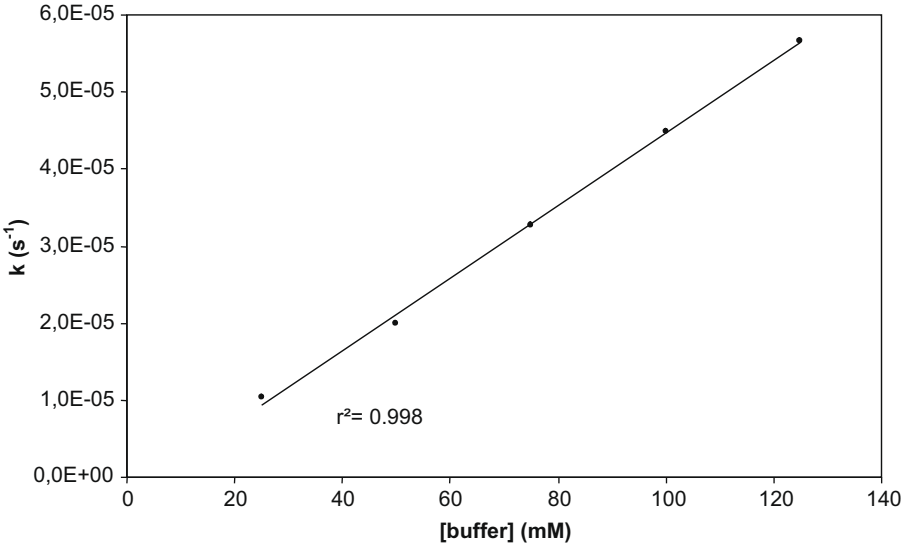

Figure 3. Influence of the phosphate buffer concentrations (pH 2.5) on the rate constant of the racemization of the (−)-9-OHRisp at 37 °C.

Table 3
Kinetic parameters (k_{rac} and $t_{1/2\,rac}$) and free energy barriers ($\Delta G^{\neq}$) for the racemization of (−)-9-OHRisp in 100 mM phosphate buffers pH 2.5 or pH 3.5: influence of the temperature

pH	T (°C)	k_{rac} (s^{-1})	$t_{1/2\,rac}$ (h)	$\Delta G^{\neq}$ (kJ mol^{-1})	$\ln k/T$ (s^{-1} K^{-1})
2.5	10	1.77×10^{-6}	109	104.2	−18.89
	10[a]	1.10×10^{-6}	98	101.5	−19.37
	25	1.27×10^{-5}	15.2	100.9	−16.97
	30	2.21×10^{-5}	8.7	101.2	−16.43
	37	4.47×10^{-5}	4.3	101.8	−15.75
	45	1.09×10^{-4}	1.8	102.8	−14.89
3.5	25	4.38×10^{-6}	43.9	103.6	−18.04
	30	7.56×10^{-6}	25.5	104.0	−17.51
	37	1.85×10^{-5}	10.4	104.1	−16.63
	45	3.74×10^{-5}	5.1	105.0	−15.96

Mean values calculated from three experiments.
[a] 100 mM deuterated phosphate buffer.

terms are not significantly different for pH 2.5 and pH 3.5 and no difference in the mechanistic information can be assessed from these results.

2.1.3. Influence of co-solvent

The influence of the addition of an organic co-solvent on the kinetics of racemization at 37 °C was investigated by adding 10%, 20%, and 40% (%v) of methanol or ethanol in 100 mM phosphate buffers pH 2.5. To avoid electrophoretic problems (falls of current), twofold or fourfold dilutions with water were performed just before each analysis to reduce the amount of organic solvent in the injected sample (10% maximum). As seen in Table 4, the addition of a large amount of methanol or ethanol (higher than 10% (v)) involves a significant increase in the racemization rate. However, by comparing the results obtained with methanol and ethanol, it appears that the nature of the alcohol has very little influence on the racemization rate. The fastest racemization observed by adding MeOH or EtOH can be explained by their higher H-bond basicity than by adding water (the H-bond acceptor property β of MeOH, EtOH, and water are 0.66, 0.75, and 0.47, respectively).[16]

2.1.4. Influence of cyclodextrins

Cyclodextrins (CDs), cyclic oligosaccharides, possess a hydrophobic cavity and a hydrophilic outer surface. These properties make them complexation agents of interest since they can include a great variety of molecules of appropriate polarity and dimension.[17] The ability of CDs to influence the racemization process of various pharmaceutical compounds has already been reported.[10–12] In our previous work, the complexation of the 9-OHRisp with a wide variety of CDs was studied using CE and NMR: at pH 2.5, the β-CD, methyl-β-CD, and hydroxypropyl-β-CD provide the highest complexation with binding constants of 165, 138, and 85 M^{-1}, respectively.[18] Thus, the influence of these three CDs on the racemization of the (+)-9-OHRisp and (−)-9-OHRisp in the 100 mM phosphate buffer pH 2.5 at 37 °C was investigated. The addition of the CDs for the 5:1, 10:1, and 100:1 M ratios has no influence since similar kinetic parameters were obtained with or without CDs whatever the enantiomer studied. For example, the k_{rac} determined without CD and in the presence of β-CD, methyl-β-CD, or hydroxypropyl-β-CD (ratio 5:1) are 4.47×10^{-5} s^{-1}, 4.63×10^{-5} s^{-1}, 4.58×10^{-5} s^{-1}, or 4.37×10^{-5} s^{-1}. As it was previously shown, the interaction between the 9-OHRisp and the CDs occurs through an inclusion of the phenyl ring in the hydrophobic CD cavity.[18] The lack of influence of the CD on the racemization rate can thus perhaps be explained by a too large distance between the CD and the stereogenic center.

Table 4
Kinetic parameters (k_{rac} and $t_{1/2\,rac}$) and free energy barriers ($\Delta G^{\neq}$) for the racemization of (−)-9-OHRisp at 37 °C: influence of the addition of organic solvent in the 100 mM phosphate buffer pH 2.5

Organic solvent	k_{rac} (s^{-1})	$t_{1/2\,rac}$ (h)	$\Delta G^{\neq}$ (kJ mol^{-1})
No solvent	4.47×10^{-5}	4.3	101.8
EtOH 10% (%v)	4.41×10^{-5}	4.4	101.9
EtOH 20% (%v)	4.94×10^{-5}	3.9	101.6
EtOH 40% (%v)	5.55×10^{-5}	3.5	101.3
MeOH 10% (%v)	4.40×10^{-5}	4.4	101.9
MeOH 20% (%v)	4.83×10^{-5}	4.0	101.6
MeOH 40% (%v)	5.20×10^{-5}	3.7	101.4

Mean values calculated from three experiments.

2.2. Mechanism of racemization

By considering the chemical structure of the 9-OHRisp and more especially the presence of an acidic hydrogen attached to the β-carbon of the nitrogen of the imine function, an imine–enamine tautomerism was supposed. The existence of this tautomerism, catalyzed in both basic and acidic conditions, could explain the racemization phenomenon since this hydrogen is precisely located at the stereogenic center (H_9). NMR experiments in D_2O were then performed to study the possible lability of H9. In the literature, the

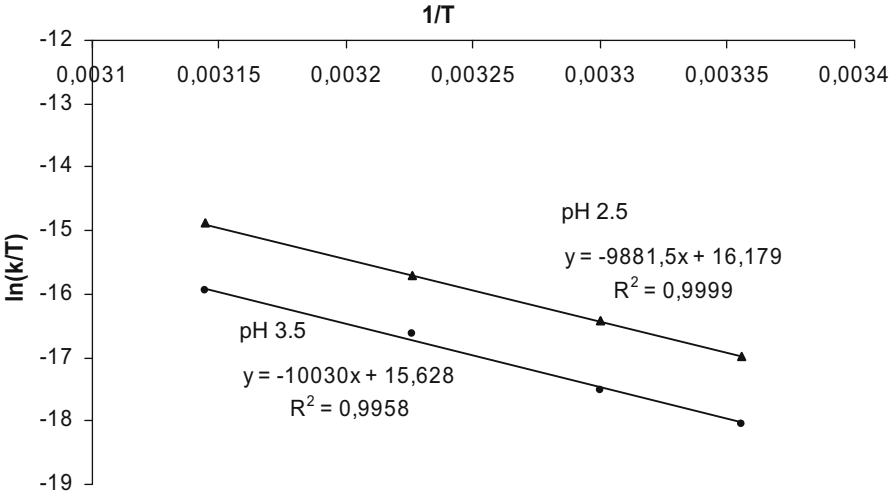

Figure 4. Thermodynamic study: Influence of the temperature on the rate constant of the racemization of the (−)-9-OHRisp in 100 mM phosphate buffers pH 2.5 or pH 3.5.

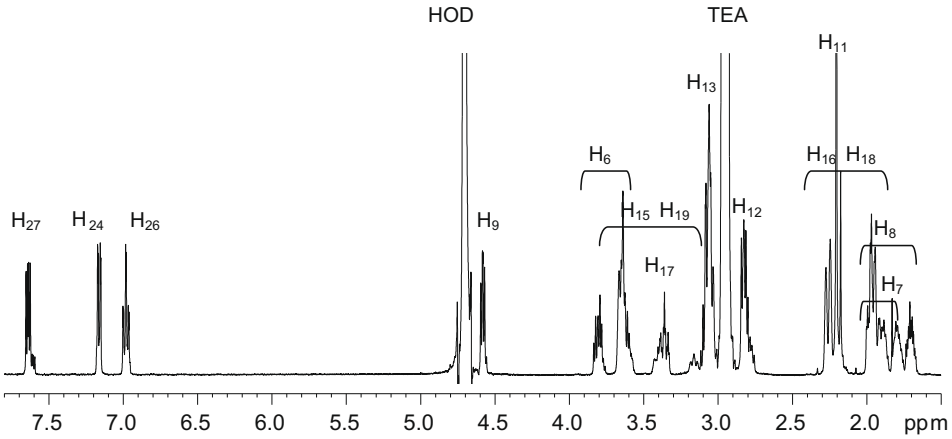

Figure 5. ^{1}H NMR spectrum of (±)-9-OHRisp (8 mM in a 100 mM phosphate buffer pD 2.5 at 10 °C).

^{1}H spectra of 9-OHRisp and Risp in chloroform $CDCl_3$ are partly described.[19] In this study, the total attribution of the ^{1}H signals was performed in D_2O (100 mM phosphate buffer pD 2.5), the results are displayed in Figure 5.

Since the fastest racemizations of the 9-OHRisp were observed at pH 2.5, the study of its deuteration (H/D exchange) was performed using an acidic phosphate buffer pH 2.5 (100 mM). Contrary to the racemization study, the deuteration can be investigated using the racemic 9-OHRisp. The temperature was selected by taking into account two phenomena. First, the deuteration of the analyte during the acquisition of the spectra (around 5 min) must be negligible. Second, at 25 °C, the signal of the interesting H_9 of the 9-OHRisp was masked by the water signal; this last signal must be shifted with the temperature. Finally, the temperature of 10 °C was chosen as a good compromise.

For a first-order H/D exchange reaction, the linear $\ln I_t/I_0$ versus time plot (with I_t, the integral of the exchanging proton at time t and I_0, the integral of the exchanging proton at time 0) permits us to determine the apparent rate constant and half-life time of the deuteration (k_{deut} = −slope and $t_{1/2\,deut} = \ln 2/k_{deut}$) (Eq. 6).

$$\ln\left(\frac{I_t}{I_0}\right) = -k_{deut}t \tag{6}$$

Since the plot obtained is linear (r^2 = 0.985), the H/D exchange is of first-order. The calculated $t_{1/2\,deut}$ is of 121 h. The isotopic exchange of the H_9 is clearly displayed in Figure 6. The kinetics of deuteration

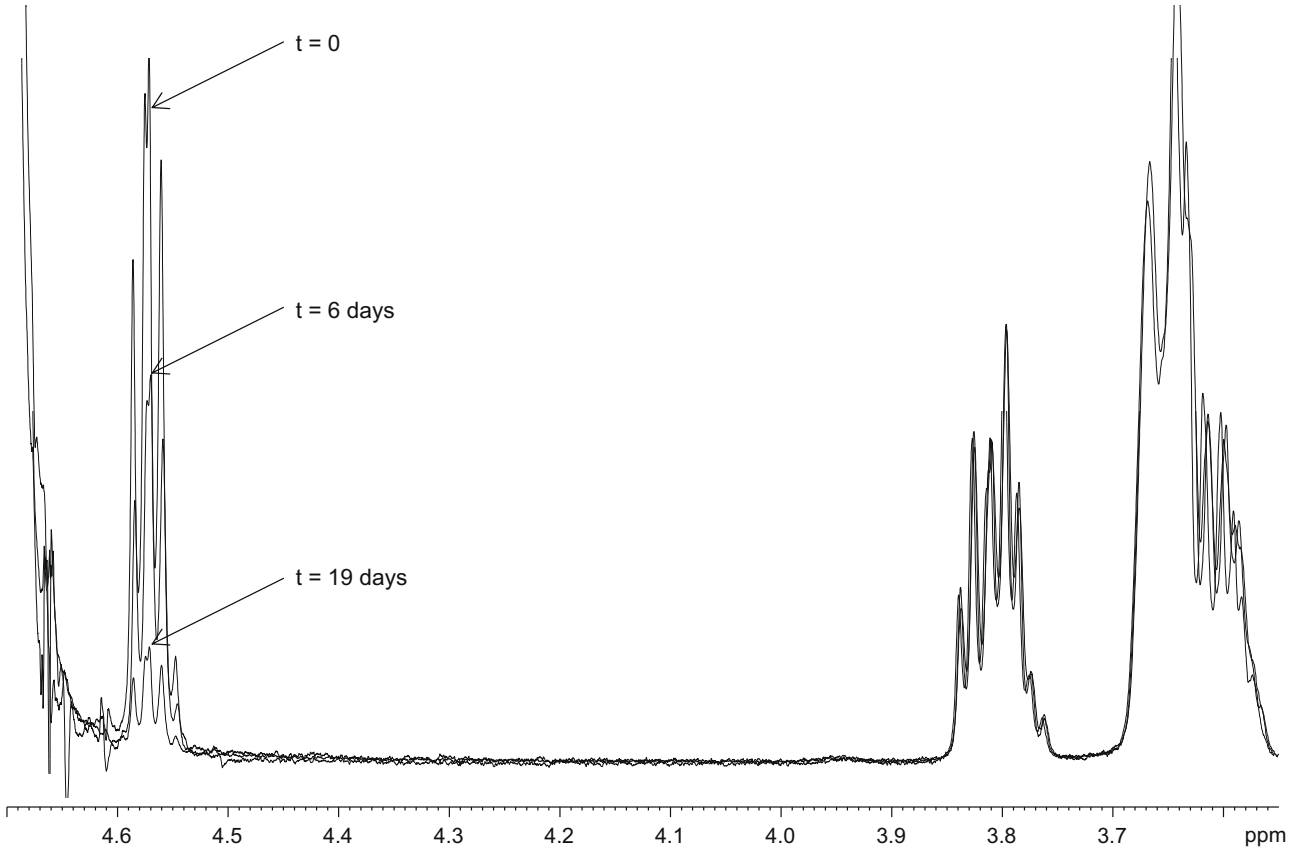

Figure 6. H/D exchange of H_9: extract of the ^{1}H NMR spectra of the (±)-9-OHRisp after 0, 6 days and 19 days (8 mM in a 100 mM phosphate buffer pD 2.5 at 10 °C).

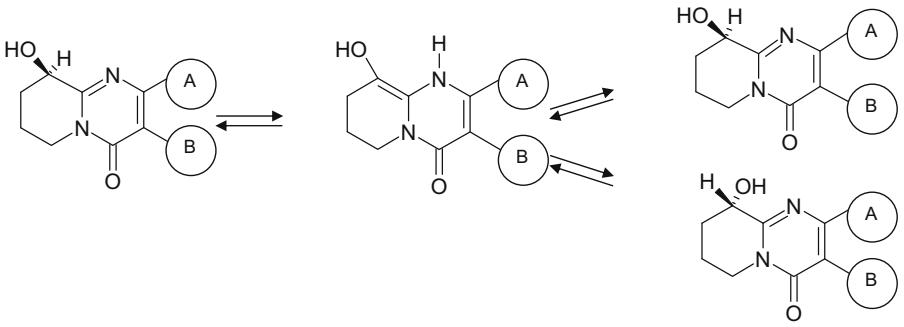

Figure 7. Imine–enamine tautomerism catalyzed in acid or basic conditions.

were compared with the kinetic of racemization and, to avoid any solvent isotope effect, the racemization of 9-OHRisp in the NMR conditions (10 °C) was investigated by CE in both deuterated and non-deuterated solvents: the $t_{1/2\,rac}$ obtained are 98 and 109 h, respectively (Table 3). A small isotopic effect on the racemization rate was then observed but the deuteration and racemization, investigated in the same deuterated buffer, show similar kinetic. Since the k_{deut}/k_{rac} ratio approaches unity, the deuteration occurs with complete racemization and both deuteration and racemization share a common mechanism.[20] It is worth mentioning that the lability of the H_9 of the achiral Risp was also displayed by the study of its H/D exchange (with faster kinetics than that observed for its metabolite).

All these results confirm that the racemization occurs via an imine–enamine tautomerism as proposed in Figure 7 and agree with the various results of the racemization study.

3. Conclusion

Strongly acidic conditions (or strongly basic conditions to a lesser extent) favor the racemization of 9-OHRisp. The proposed mechanism is supported by the presence of the proton–deuterium exchange of the proton borne by the chiral carbon and suggests an imine–enamine tautomerism.

Since it was proved that racemization does not occur under the physiological conditions, the stereoselectivity of the transport of the isolated enantiomers of 9-OHRisp across Caco-2 cell monolayers (in vitro model for intestinal drug absorption) could be studied using a faster achiral analytical method.

4. Experimental

4.1. Reagents and solutions

The racemic 9-hydroxyrisperidone (9-OHRisp) was a gift from Janssen-Cilag (Issy-les-Moulineaux, France). Both enantiomers were isolated by preparative HPLC enantioseparation as previously described using a cellulose chiral stationary phase (Chiralcel OJ) in the normal phase mode. Their enantiomeric purity, quantified using a validated analytical method, is superior to 99.9% or equal to 98.9% for the dextrorotatory or levorotatory enantiomers, respectively.[13] Deuterium oxide (100%) was purchased from Euriso-top (Gif sur Yvette, France). Methanol (MeOH), ethanol (EtOH), phosphoric acid (85% w/w), boric acid, sodium hydroxide (NaOH), sodium chloride (NaCl), triethylamine, triethanolamine (TEA), and tris-(hydroxymethyl)aminomethane (Tris) were purchased from Merck (Nogent-sur-Marne, France). Deionized water was obtained from a Milli-Q system (Millipore, Saint-Quentin en Yvelines, France). The phosphate buffers were prepared from a phosphoric acid solution adjusted to pH 2.5 by addition of triethanolamine; an appropriate volume of sodium chloride (1 M) was occasionally added in order to set the ionic strength to the desired value (0.1 M). The borate buffer was prepared from acid boric and the pH was adjusted by addition of sodium hydroxide (5 M). The Tris buffer was prepared by dissolving an appropriate amount of Tris and the pH was adjusted to 7.4 by the addition of a phosphoric acid solution (0.1 M). β-CD, HP-β-CD, and Me-β-CD were a gift from the Roquette Laboratories (Lestrem, France). The modified cyclodextrins 2-hydroxypropylated-β-CD (HP-β-CD) and methylated-β-CD (Me-β-CD) represent multicomponent mixtures with molar substitution (MS) 0.75–0.95 and 0.5 per anhydroglucose unit, respectively; the sulfated-α-CD (S-α-CD) with MS 1–1.8 per anhydroglucose unit was purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). The molar concentrations of these substituted CDs were calculated taking into account their averaged molecular weight.

4.2. Capillary electrophoresis

CE experiments were performed on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Villepinte, France), including an on-column diode-array UV-detector. The whole system was driven by a personal computer with the 32 Karat software package for system control, data collection, and analysis. It was equipped with a 50.2 cm (40 cm effective length) × 50 μm I.D. untreated fused-silica capillary (Composite Metal Services, Ilkley, UK). The capillary was mounted on a cartridge and thermostated at 298 K ± 0.1 K and the applied voltage was 20 kV. The hydrodynamic injections were made with a 5 s injection time at 1.0 psi pressure. The 9-OHRisp was detected simultaneously at 190, 234, and 270 nm. New capillaries were flushed for 20 min with 0.1 M NaOH (P = 25 psi) and 5 min with water (P = 25 psi). Each day the capillary was flushed successively with NaOH (5 min, 25 psi), water (1 min, 25 psi), and then with background electrolyte (BGE) (3 min, 25 psi). Between each run, it was treated with water (1 min, 25 psi) and BGE (2 min, 25 psi).

The CE used method for the enantioseparation of 9-OHRisp had previously been developed and validated.[13,14] The background electrolyte was composed of an 80 mM phosphate buffer pH 2.5, HP-β-CD (37 mM), and S-α-CD (3.7% (w/v)); the voltage and temperature were 20 kV and 25 °C, respectively. Under these conditions, both enantiomers were resolved in an analysis time of about 13 min with an enantioresolution of 3.13.

4.3. Kinetic of racemization

The kinetic experiments were conducted in the temperature controlled sample storage unit of the CE apparatus. The temperature controlled sample storage unit allows to maintain constant the temperature of the samples along the racemization process

and moreover permits easy automation. The analyte concentration was 0.20 mM in all cases. Each kinetic result was triplicated; all the displayed results are mean values.

4.4. Kinetic of the H/D exchange

The NMR spectroscopy experiments were performed on a Bruker Avance 500 with a TXI probe operating at 500.13 MHz and the data were analyzed using the Topspin software. The attributions of the ^{1}H signals of Risp and 9-OHRisp were performed by various NMR experiments: COSY (correlation spectroscopy) (correlation homonuclear ^{1}H–^{1}H), HSQC (heteronuclear single quantum coherence) (correlation heteronuclear ^{1}H–^{13}C) and HMBC (heteronuclear multiple bond correlation) (correlation long range ^{1}H–^{13}C). 500 μL of solutions (8 mM in the deuterated 100 mM phosphate buffer, pD 2.5) were introduced into a standard 5 mm NMR tubes. For the study of the H/D exchange, spectra were recorded regularly for one month at 283 K. The temperature control of the probe was approximately ±0.5 °C. Thirty two scans of 32k data points were acquired with a spectral width of 5000 Hz. The FIDs were transformed with a line broadening of 0.3 Hz. The kinetics of the H/D exchange were investigated by integration of the signal of the exchanging proton H_9 at initial time and at *t* time (the calibration of integrals was performed from the signal of an unexchangeable reference proton H_{24}).

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