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Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 17 (2006) 2317–2321

Kinetics of racemization of enantiopure *N*-imidazole derivatives, aromatase inhibitors: studies in organic, aqueous, and biomimetic media

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Received 5 July 2006; revised 15 August 2006; accepted 20 August 2006

Abstract—The configurational stability of *N*-imidazole derivatives, aromatase inhibitors, was investigated under organic, aqueous, and biomimetic media, by studying the effect of organic solvent, pH, and temperature. Validated chiral HPLC or CD-EKC methods allowed us to quantify the presence of both enantiomers. The rate constants, half-lives, and apparent free energy barriers of the racemization phenomenon were determined using a mathematical model of this first-order reaction.

The presence of triethylamine (2000 equiv) at high temperature (70 °C) induces the fastest racemization with a $t_{1/2\,rac}$ less than 7 h. This result can be related to the lability of benzylic proton in these strongly basic conditions. Biomimetic media (the presence of bovine serum albumin in a pH 7.4 buffer at 37.4 °C) do not seem to preserve the initial configuration of each enantiomer.
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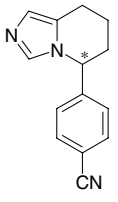
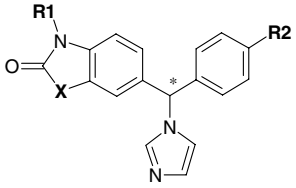
1. Introduction

Since enantiomers often differ in pharmacological activity, toxicity, and pharmacokinetic characteristics, it is important to evaluate drug candidates in enantiomerically pure forms whenever possible.[1,2] One major problem arising from the presence of elements of chirality is their possible lack of configurational stability, that is, the danger of interconversion of stereoisomeric drugs. Hence during the discovery stage of new chiral compounds, it is of interest to determine the pharmacokinetics and possible inversion of the enantiomers.[3] Two time scales and related sets of conditions are relevant, as far as the drugs are concerned. The pharmaceutical time scale implies that drugs remain configurationally stable during the whole manufacturing process and for its shelf-life. The pharmacological time scale is concerned with stability under physiological conditions (37 °C, pH 7.4) and for the time of residence of the drug in the body.[4]

The *N*-imidazole derivatives studied are new 3-methyl-6-[1-(imidazo-1-yl)-1-phenylmethyl] benzothiazolinones **1** and **3** and 3-methyl-6-[1-(imidazo-1-yl)-1-phenylmethyl] benzoxazolinones **2** and **4** derivatives (Fig. 1) synthesized[5,6] as potential aromatase inhibitors, useful in the treatment of estrogen dependant breast cancer in postmenopausal women.[7] The in vitro activities of these



Fadrozole



Compound	X	R1	R2
1	S	CH_3	H
2	O	CH_3	H
3	S	CH_3	CN
4	O	CH_3	CN

Figure 1. Aromatase inhibitors **1**–**4**.

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doi:10.1016/j.tetasy.2006.08.014

compounds were tested in human and equine aromatase and many of them present higher activity than the reference compound fadrozole.[8] Since pharmacological studies have shown that enantiomers of aromatase inhibitors may differ in activity, metabolism or toxicity, for example, (*S*)-fadrozole is the active enantiomer,[9] a chiral preparative separation was necessary to test the individual enantiomers of these compounds.

Our approach of the chiral separation of these analytes began with the screening of a number of chiral stationary phases by high performance liquid chromatography, which revealed high enantioselectivity to separate the enantiomers.[10,11] However, in the first step, analysis of the isomers after evaporation of the solvents (to check their enantiomeric purity) has shown the presence of the undesired enantiomer. Comparison of the enantiomeric purities, before and after rotary evaporation (bath temperature 40–50 °C), revealed a significant racemization in the hexane/ethanol mobile phase at this temperature. This type of phenomenon was previously described by Welch et al.[12] and Caccamese et al.[13] Nevertheless, in a second step, sufficient purified individual enantiomers carefully obtained by preparative HPLC (after evaporation at room temperature)[14] allowed us to determine the absolute configutation for **1**–**4** and to study the pharmacological behavior.

Herein, the kinetics of the racemization of these recently discovered aromatase inhibitors **1**–**4** was established in various organic, aqueous and biomimetic media, studying the effect of the nature of the organic solvents, temperature, pH and addition of serum albumin. The enantiomeric excess (ee) was determined by using previously validated chiral HPLC methods[14] and chiral CE methods developed using cyclodextrins.[15,16]

2. Results and discussion

The racemization phenomenon that occurs in organic media was studied by previous HPLC validated enantioseparation methods[14] using polysaccharide-derived chiral stationary phases (cellulose and amylose) in normal phase (hexane–ethanol). However, in order to avoid any sample preparation, such as extraction, chiral capillary electrokinetic validated methods[15] using cyclodextrins (CD-EKC with highly Sulfated-β-CD) were preferred to HPLC methods for aqueous and biomimetic media.

The enantiomeric excesses were calculated from the concentration of each enantiomer according to their respective UV signal. A linear plot of ln ee versus time indicates first-order kinetic of racemization and allows us to determine the apparent constant rates ($k_{rac} = -\text{slope}$) and half-lives ($t_{1/2\,rac} = \ln 2/k_{rac}$), defined as the time required for an enantiopure solution to reach 50% ee.[17,18] The apparent free energy barrier ($\Delta G^{\neq}$) of racemization is calculated according to the Eyring equation:[19,20]

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

(with $R = 8.314\ \text{J mol}^{-1}\ \text{K}^{-1}$, $h = 6.626 \times 10^{-34}\ \text{J s}$ and $k_b = 1.381 \times 10^{-23}\ \text{J K}^{-1}$)

In order to replicate the initial conditions of racemization, the enantiomeric stability was first studied for **1**–**4** (first batch for **4**) in pure ethanol and in a hexane/ethanol 50/50 mixture at 50 °C. Since no racemization was observed after 4 days, the enantiomeric instability detected first was probably induced by impurities in the solvents used for the preparative enantioseparation.

The effect of acid (acetic acid) or base [triethylamine (TEA)] addition at various concentrations in ethanolic solution on the chiral stability at 50 °C was studied for compounds **1**–**4**. It appears that whatever the conditions, no racemization occurs for **1** and **2**. For **3** and **4**, racemization occurs only with base addition (3000–100 equiv), whereas the acid additions (3000–1000 equiv) had no effect. The kinetic parameters (k_{rac} and $t_{1/2\,rac}$) and apparent free energy barriers ($\Delta G^{\neq}$) obtained for (−)-**3** and (−)-**4** in basic conditions are listed in Table 1. The data reveal that the rate constant increases with the TEA/enantiomer ratio up to 2000 equiv (after which it remains roughly constant) with $t_{1/2\,rac}$ of 12.4 and 16.4 h for (−)-**3** and (−)-**4**, respectively. As expected, the decrease of the $t_{1/2\,rac}$ is correlated with a decrease of the activation energy barrier: a $\Delta G^{\neq}$ significant difference of 1.71 kJ mol^{-1} is calculated for TEA/enantiomer ratios between 100 and 2000. Figures 2 and 3 show the chromatograms and a plot of ln ee versus time obtained for (−)-**4** in the presence of TEA (2000 equiv).

Table 1. Kinetic parameters (k_{rac} and $t_{1/2\,rac}$) and free energy barriers ($\Delta G^{\neq}$) for the racemization of the (−)-**3** and (−)-**4** enantiomers in EtOH/TEA at 50 °C (analytical HPLC conditions as in Fig. 2)

Compound	Diluent (equiv)	k_{rac} (s^{-1})	$t_{1/2\,rac}$ (h)	$\Delta G^{\neq}$ (kJ mol^{-1})
(−)-**3**	EtOH/TEA (100)	8.18 (±0.12) 10^{-6}	23.6 ± 0.7	110.78 ± 0.04
	EtOH/TEA (1000)	1.40 (±0.04) 10^{-5}	13.7 ± 0.3	109.34 ± 0.03
	EtOH/TEA (2000)	1.55 (±0.03) 10^{-5}	12.4 ± 0.3	109.07 ± 0.02
	EtOH/TEA (3000)	1.59 (±0.04) 10^{-5}	12.1 ± 0.3	109.01 ± 0.02
(−)-**4**	EtOH/TEA (100)	4.29 (±0.09) 10^{-6}	43.8 ± 0.9	112.51 ± 0.05
	EtOH/TEA (1000)	1.09 (±0.02) 10^{-5}	17.7 ± 0.3	110.01 ± 0.04
	EtOH/TEA (2000)	1.17 (±0.03) 10^{-5}	16.4 ± 0.4	109.81 ± 0.03
	EtOH/TEA (3000)	1.21 (±0.03) 10^{-5}	15.9 ± 0.3	109.73 ± 0.02

Mean values for three experiments.

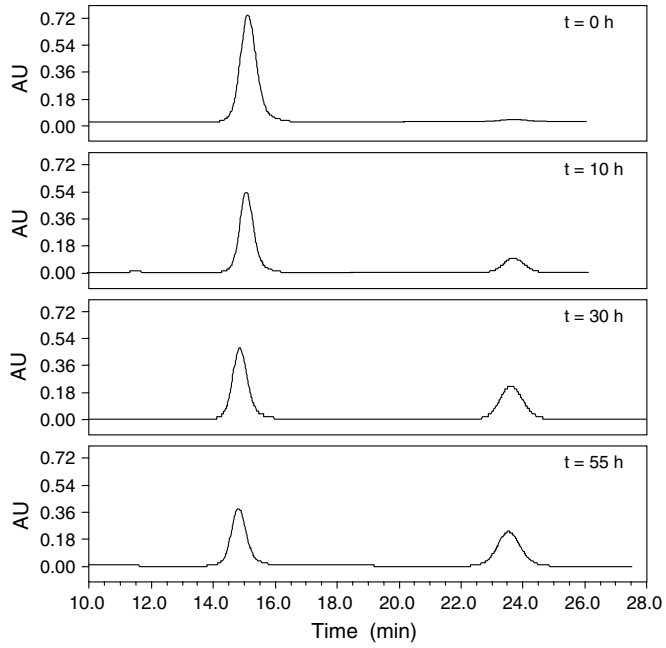

Figure 2. Stacking chromatograms of (−)-**4** after 0, 10, 30 and 55 h in ethanol with TEA (2000 equiv) at 50 °C (analytical HPLC conditions: Chiralpak AD; eluent: hexane/ethanol (40/60, v/v); temperature 30 °C; flow rate: 0.8 mL min^{-1}).

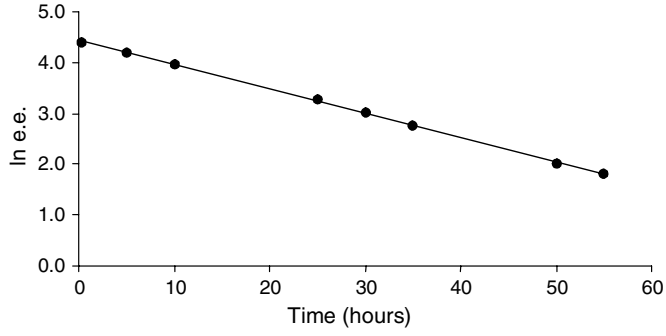

Figure 3. Plot of ln ee versus time (in the range 0–55 h) obtained for (−)-**4** in ethanol with TEA (2000 equiv) at 50 °C.

Moreover, both enantiomers of these two compounds were shown to have identical kinetics of chiral inversion.

The chiral inversion mechanism could occur through base-induced deprotonation of the labile benzylic proton borne by the stereogenic carbon (C_8) that leads to a planar carbanion, while the following readdition of the removed proton may occur at random and favors formation of the racemic compound. The greater chiral inversion of **3** and **4** is explained by the greater acidity of the proton at C_8, due to the presence of a strong electron withdrawing cyano group on the phenyl ring.

The influence of the organic solvent on the racemization was studied for (+)-**4** and (−)-**4**: ethanol was changed to dimethylsulfoxide, acetonitrile or methanol, adding in all cases 2000 equiv of TEA in the solvent. The nature of the organic solvent seems to be important with respect to the racemization phenomenon: when DMSO and acetonitrile are used, the $t_{1/2\,rac}$ increased by 200% and 1862%, respectively, whereas no racemization was observed after 5 days when methanol is employed. Once again, the lower $\Delta G^{\neq}$ was determined under the conditions that permitted the fastest racemizations. No correlation between the racemization rate and the polarity of the solvent was found. However, taking into account the H-bond acceptor property of the solvents,[21] it appears that the higher H-bond acceptor capacities may be correlated to higher racemization rates (Table 2).

Since temperature is recognized as an important parameter affecting the racemization rate, its influence was studied for (−)-**4**. As expected, experimental data revealed that the chiral inversion is greatly influenced by the temperature (Table 3): the corresponding $t_{1/2\,rac}$ obtained with a temperature of 30, 50, or 70 °C are 117.8, 15.4, or 6.6 h, respectively. The thermodynamical parameter $\Delta G^{\neq}$ increases with temperature (in accordance with the Van't Hoff equation) and its associated racemization: $\Delta G^{\neq}$ are 107.83, 109.81, or 114.19 kJ mol^{-1} for 30, 50, or 70 °C, respectively.

Since racemizations at physiological pH of drug enantiomers bearing a labile proton at the chiral center have already been shown,[4,22,23] and since strongly basic conditions in organic media were found to favor the racemization of these aromatase inhibitors, the enantiomeric stabilities of these compounds were studied in aqueous media at physiological and basic pH.

CD-EKC analyses revealed that only compounds **3** and **4** were enantiomerically unstable in a 50 mM phosphate buffer or 50 mM TRIS buffer at pH 7.4 and in a 50 mM phosphate buffer at 9.6 (with 10% of ethanol to permit analyte solubilization). Once again, an identical kinetic behavior of racemization was observed for both levorotatory and

Table 2. Kinetic parameters (k_{rac} and $t_{1/2\,rac}$) and free energy barriers ($\Delta G^{\neq}$) of the racemization phenomenon of the (−)-**4** in various organic solvents with TEA (2000 equiv) at 50 °C (Analytical HPLC conditions as in Fig. 2)

Solvent (with TEA 2000 equiv)	β[a]	k_{rac} (s^{-1})	$t_{1/2\,rac}$ (h)	$\Delta G^{\neq}$ (kJ mol^{-1})
EtOH	0.75	1.17 (±0.03) 10^{-5}	16.4 ± 0.4	109.81 ± 0.03
DMSO	0.76	3.91 (±0.08) 10^{-6}	49.2 ± 1.2	112.76 ± 0.05
CH_3CN	0.40	5.98 (±0.01) 10^{-7}	321.8 ± 5.1	117.81 ± 0.07
MeOH	0.66	No racemization after 4 days		

Mean values for three experiments.
[a] β: H-bond basicity, measure of the H-bond acceptor property.[21]

Table 3. Kinetic parameters (k_{rac} and $t_{1/2\,rac}$) and free energy barriers ($\Delta G^{\neq}$) for the racemization of the (−)-**4** enantiomer in ethanol with TEA (2000 equiv) at various temperatures (Analytical HPLC conditions as in Fig. 2)

Temperature (°C)	k_{rac} (s^{-1})	$t_{1/2\,rac}$ (h)	$\Delta G^{\neq}$ (kJ mol^{-1})
30	1.63 (±0.01) 10^{-6}	117.8 ± 2.5	107.83 ± 0.03
50	1.17 (±0.03) 10^{-5}	16.4 ± 0.4	109.81 ± 0.03
70	2.92 (±0.06) 10^{-5}	6.6 ± 0.2	114.19 ± 0.05

Mean values for three experiments.

dextrorotatory enantiomers and the kinetic increases in strong basic conditions. The nature of the buffer, phosphate or TRIS, has no effect on the racemization rate. The $t_{1/2\,rac}$ is approximately 10 times weaker at pH 7.4 than at pH 9.6, they are for **3** (1988 and 204 h) and for **4** (2832 and 270 h). Figures 4 and 5 present the electropherograms and the plot of ln ee versus time of (−)-**4** after 0.25, 30, and 80 h in the pH 9.6 solution.

In order to approach the physiological media, bovine serum albumin (BSA) was added in aqueous solution at pH 7.4 (7 g L^{-1} in the 50 mM phosphate buffer) and its influence on the racemization was studied at 37 and 50 °C for both enantiomers of **4**. Electropherograms of (−)-**4** after 0.25 and 80 h at 50 °C are presented in Figure 6 and show

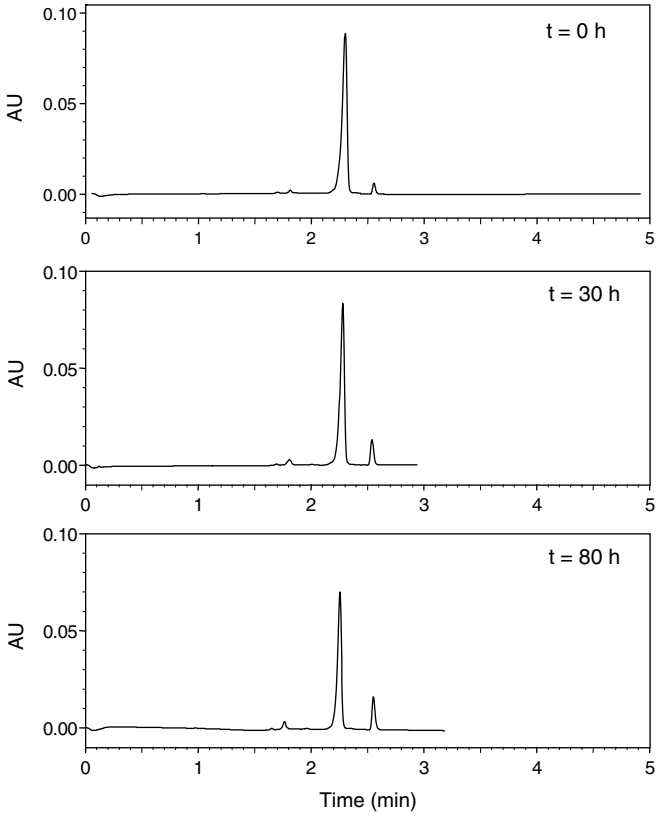

Figure 4. Stacking electropherograms of (−)-**4** after 0, 30 and 80 h in a 50 mM phosphate buffer at pH 9.6 (with 10% ethanol) at 50 °C [analytical CZE conditions: BGE, 25 mM phosphate buffer, pH 2.5 (H_3PO_4 + TEA); [highly S-β-CD] = 3% (w/v)].

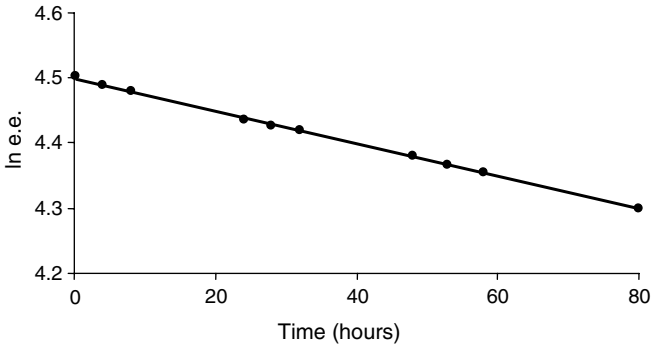

Figure 5. Plot of ln ee versus time (in the range 0–80 h) obtained for (−)-**4** in a 50 mM phosphate buffer at pH 9.6 (with 10% ethanol) at 50 °C.

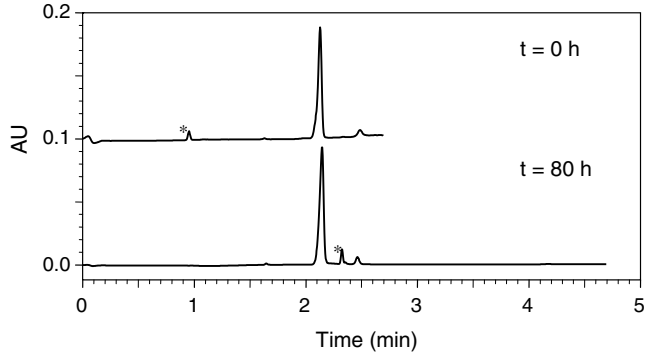

Figure 6. Stacking electropherograms of (−)-**4** after 0 and 80 h in a 50 mM phosphate buffer at pH 7.4 containing BSA at 7 g L^{-1} at 50 °C (with 10% ethanol) [analytical CZE conditions: BGE, 25 mM phosphate buffer, pH 2.5 (H_3PO_4 + TEA); [highly S-β-CD] = 3% (w/v)] (*: impurities).

the specificity of the separation. Since a similar racemization rate was obtained with or without the addition of BSA in the phosphate buffer, pH 7.4, BSA seems to have no effect on the racemization kinetics: BSA does not seem to permit the preservation of the initial configuration of the enantiomers under these weak racemizing conditions.

3. Conclusion

We have shown that some of the **1**–**4** analytes possessing a benzylic proton at the stereogenic center isomerize under certain conditions. This may be an important point since the aromatase inhibitory potency of these molecules possessing a stereogenic center may mainly reside in one enantiomer, as it has been pointed out for fadrozole. This observation of the lability of the benzylic type C–H at the stereogenic center in enantiomers is essential for avoiding unintentional racemization during the synthesis, storage and analytical procedures: it will be necessary to prohibit strongly basic conditions. This observation could also be essential for limiting the tissue and animal experimentation programs or the chiral synthesis, with its associated development costs, at the early stage prior to submission for drug licensing since initial pure enantiomers may racemize during the processes.

4. Experimental

4.1. Reagents and solutions

Analytes **1**–**4** were prepared according to the synthetic route previously described,[5,6,8] leading to a racemic mixture of enantiomers. Both enantiomers of **1**–**4** were isolated through the preparative HPLC enantioseparation methods previously described.[14] Their purity was quantified using the validated analytical methods.[14] In a first step, the isolation of (−)-**4** and (+)-**4** without precaution (evaporation at bath temperature of 40–50 °C) leads to enantiomeric purities of 98.76% and 97.54%, respectively (first batch). In a second step, careful isolations (evaporation at room temperature) of **1**–**4** furnished the enantiomers with larger enantiomeric purities:[14] 99.81% for (+)-**1**, 99.48% for (−)-**1**, 99.88% for (+)-**2**, 99.36% for (−)-**2**, 99.79% for (+)-**3**, 99.08% for (−)-**3**, 99.18% for (+)-**4** and 99.27% for (−)-**4** (second batch for **4**).The first batch of (−)-**4** and (+)-**4** was used here for the racemization study.

Bovine Serum Albumin (BSA), fraction V, was purchased from Sigma–Aldrich (Lyon, France). Triethylamine (TEA) was obtained from Acros (Geel, Belgium). Ethanol, methanol, acetonitrile and tri-(hydroxymethyl)aminomethane (TRIS) were purchased from Merck (Nogent-sur-Marne, France).

4.2. Kinetic studies on racemization

The analyte concentration was 0.40 mM in all cases. All kinetic experiments were conducted in a closed vessel immersed in a water bath. The temperature (30, 37, 50, or 70 °C) was monitored using a calibrated digital thermometer. The samples were withdrawn at fixed time intervals, stored at 0 °C before analyses, passed through a 0.45 μm membrane filter and analyzed either by HPLC (for organic media) or by Capillary Electrophoresis (for aqueous and biomimetic media). For the samples containing BSA, the protein was precipitated by two volumes of ethanol before filtration. This rapid sample preparation permits us to eliminate the most part of BSA. The specificity has been verified by injection of the matrix (without analyte) after identical precipitation and filtration: no interference was detected. Each study was triplicated and all results expressed are mean values.

4.3. Chiral liquid chromatography

Analytical chiral chromatography was performed on a Chiralpak AD amylose column (tris-3,5-dimethylphenylcarbamate; 250 × 4.6 mm i.d.; 10 μm) or on a Chiralcel OD-H cellulose column (tris-3,5-dimethylphenylcarbamate; 250 × 4.6 mm i.d.; 5 μm) (Daicel Chemical Industries, Baker France). A constant mobile phase flow of 0.8 mL min^{-1} was provided by a gradient Waters 600E metering pump model equipped with a 7125 Rheodyne injector (20 μL loop). Detection was achieved with a Waters 996 photodiode array spectrophotometer at the maximum wavelengths that are, respectively 224 nm for **1**, 203 nm for **2**, 220 nm for **3**, and 205 nm for **4**. Chromatographic data were collected and processed on a computer running with Empower software. Chromatography was performed at 30 °C.

4.4. Capillary electrophoresis

CE experiments were performed on a Beckman P/ACE MDQ Capillary Electrophoresis system (Beckman Coulter France, Villepinte, France), including an on-column diode-array UV-detector. The whole system was driven by a PC with the 32 Karat software (Beckman Coulter France) package for system control, data collection and analysis. It was equipped with a 50.2 cm (10.2 cm effective length) × 50 μm ID untreated fused-silica capillary (Composite Metal Services, Worcestershire, UK). The capillary was mounted in a cartridge and thermostated at 298 ± 0.1 K. Highly S-α-CD, highly S-β-CD, and highly S-γ-CD (20% w/v in a 50 mM phosphate buffer at pH 2.5) were purchased from Beckman (Beckman Coulter France, Villepinte, France). Electrophoretic methods for the enantioseparation of **1**–**4** were previously described.[15]

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