Jonas Urlaub[1‡]
Reinhard P. Kaiser[2‡]
Oliver Scherf-Clavel[1]
Carsten Bolm[2]
Ulrike Holzgrabe[1]

[1]Institute for Pharmacy and Food Chemistry, University of Würzburg, Würzburg, Germany
[2]Institute for Organic Chemistry, RWTH Aachen University, Aachen, Germany

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Research Article

Investigation of isomerization of dexibuprofen in a ball mill using chiral capillary electrophoresis

Besides the racemate, the *S*-enantiomer of ibuprofen (Ibu) is used for the treatment of inflammation and pain. Since the configurational stability of *S*-Ibu in solid state is of interest, it was studied by means of ball milling experiments. For the evaluation of the enantiomeric composition, a chiral CE method was developed and validated according to the ICH guideline Q2(R1). The addition of Mg^{2+}, Ca^{2+}, or Zn^{2+} ions to the background electrolyte (BGE) was found to improve Ibu enantioresolution. Chiral separation of Ibu enantiomers was achieved on a 60.2 cm (50.0 cm effective length) x 75 μm fused-silica capillary using a background electrolyte (BGE) composed of 50 mM sodium acetate, 10 mM magnesium acetate tetrahydrate, and 35 mM heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD) as chiral selector. The quantification of *R*-Ibu in the mixture was performed using the normalization procedure. Linearity was evaluated in the range of 0.68–5.49% *R*-Ibu ($R^2 = 0.999$), recovery was found to range between 97 and 103%, the RSD of intra- and interday precision below 2.5%, and the limit of quantification for *R*- in *S*-Ibu was calculated to be 0.21% (extrapolated) and 0.15% (dilution of racemic ibuprofen), respectively. Isomerization of *S*-Ibu was observed under basic conditions by applying long milling times and high milling frequencies.

Keywords:
Capillary electrophoresis / Chiral separation / Ibuprofen / Isomerization / Validation
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1 Introduction

There are numerous chiral drugs on the market, which are administered as racemic mixtures, although pharmacological activity often resides in one enantiomer only. Its chiral antipode may show reduced activity, no activity at all, or even toxicity. The probably most widely known example of a toxic distomer is the *S*-isomer of thalidomid showing pronounced teratogenic effects [1]. Most of the β-blockers like metoprolol and bisoprolol salts are applied as racemic mixtures, although it is known that pharmacological activity is almost exclusively ascribed to one enantiomer only [2]. The same holds true for nonsteroidal anti-inflammatory drugs (NSAIDs) such as flurbiprofen and ibuprofen [3].

Ibuprofen is an NSAID with one stereogenic center at the α carbon atom. It inhibits the cyclooxygenase providing analgesic, antipyretic, and antiphlogistic effects, because it prevents the production of inflammatory prostaglandins [4,5]. Hence, ibuprofen is frequently used for the treatment of slight and moderate pain, and rheumatic diseases. The anti-inflammatory effects of Ibu can be attributed to the (+)-*S*-enantiomer (dexibuprofen), whereas (–)-*R*-Ibu is inactive (Fig. 1). Since there is a unidirectional enzymatic conversion of *R*- into *S*-Ibu *in vivo* [6–8], the racemic mixture is usually applied. However, interindividual differences in pharmacokinetics of the enantiomers might justify the use of enantiomeric pure *S*-Ibu [9,10], which was thus launched for patient's treatment also. Certainly, a compendial monograph for *S*-Ibu does not exist.

Isomerization of *S*-Ibu was already observed *in vitro* under acidic and basic conditions, where a kinetic model of

Correspondence: Prof. Dr. Ulrike Holzgrabe, University of Würzburg, Institute for Pharmacy and Food Chemistry, Am Hubland, 97074 Würzburg
E-mail: ulrike.holzgrabe@uni-wuerzburg.de

Abbreviations: **Ibu**, ibuprofen; **LAG**, liquid assisted grinding; **NSAID**, nonsteroidal anti-inflammatory drug; **Ph.Eur.**, European Pharmacopoeia; **TM-β-CD**, heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin

‡Equal contributors
Color online: See article online to view Figs. 2, 4, 5, and 7 in color.

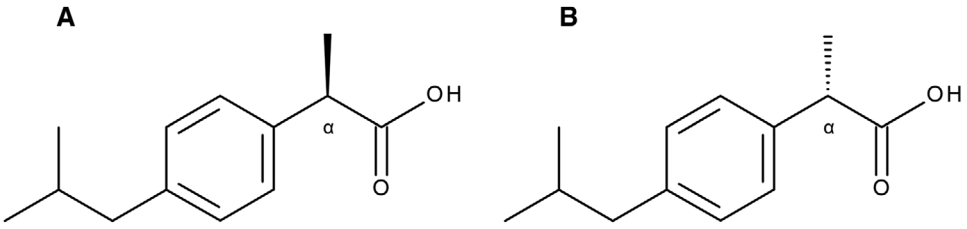

Figure 1. Inactive (–)-*R*-Ibu (A) and active (+)-*S*-Ibu (B).

base-catalyzed isomerization was evaluated [11–13]. The experiments were carried out in aqueous solution or in the melt applying high temperatures and long reaction times, including the usage of strong organic bases as isomerization additives [13]. However, the ibuprofen enantiomers were found to be highly stable under acidic conditions. Furthermore, isomerization processes in solid state have been described in the scope of asymmetric synthesis or using thermal or irradiating stress [14–19].

Mechanochemistry can offer attractive alternatives allowing a control of the reaction conditions by performing the processes in automated devices such as ball mills [20–22]. The grinding, shearing, pulling, kneading, and milling in such instruments transmits mechanical energy to reactive media inducing chemical reactions including bond-breaking and -forming processes. Many of these transformations proceed in the absence of solvent resulting in exceptionally high reactivities and leading to unusual product compositions. Recently, ball milling has been proven to be predictive for degradation processes of a drug [23]. To the best of our knowledge, mechanochemistry has not been used to investigate the isomerization of Ibu so far. Hence, the isomerization of *S*-Ibu, which was stressed in a ball mill under solid-state conditions using basic, acidic, and/or liquid additives, was studied here.

For the chiral separation of racemic Ibu by means of capillary electrophoresis, many different types of buffer, chiral selectors, capillaries, and electrophoretic procedures were reported [24–41]. Among the cyclodextrins, heptakis-(2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD) was used often [25–30]. Other β-CD derivatives like native β-CD [24,31,32] or sulfated β-CD [33] were also tested. Linear dextrins were found to act as chiral selectors as well, sometimes in combination with other chiral selectors [27,34–39]. Apart from oligosaccharides, enantiomeric separation of Ibu was also achieved using bovine serum albumin [34], vancomycin [40] or avidin [41] as chiral selectors.

As buffering agents, morpholinoethanesulfonic acid [24,26,27,31] or Tris/Phosphate [25,28,29,33] were frequently used in literature. In contrast, an acetate based BGE has been sparingly employed for Ibu enantioseparation in CE [31,32]. Moreover, a comprehensive method development using cations such as Mg^{2+} or Zn^{2+} as BGE additives has never been performed.

Up to now there is no validated CE method in literature applied on the determination of the enantiomeric purity of *S*-Ibu; reported CE methods dealing with the quantification of Ibu enantiomers are either validated [28,29,33], or tested on enantiomeric purity [37,39]. Hence, for quantitative evaluation of the *R*-Ibu content in the *S*-enantiomer after ball milling, a simple CE separation using TM-β-CD as a chiral selector and a magnesium salt as cationic additive was developed and validated as an addition to other proposed CE methods and HPLC [42].

2 Materials and methods

2.1 Chemicals and reagents

Acetic acid ≥99.8%, HPLC grade methanol, sodium hydroxide pellets, and magnesium acetate tetrahydrate ≥99% were obtained from Merck KGaA (Darmstadt, Germany). Calcium chloride hexahydrate ≥97%, magnesium chloride hexahydrate ≥99%, magnesium sulfate heptahydrate ≥99.5%, and potassium chloride ≥99.5% were purchased from Grüssing GmbH (Filsum, Germany). Zinc sulfate heptahydrate 99.7% was obtained from VWR International (Leuven, Belgium). Hydrochloric acid 37% (m/V) for preparation of CE rinsing solution and sodium chloride ≥99.5% were supplied by Bernd Kraft GmbH (Duisburg, Germany). TM-β-CD ≥ 98%, HPLC grade acetonitrile, potassium hydroxide ≥99.95%, HPLCgrade ethyl acetate ≥99.7%, HPLCgrade water used for stress tests, hydrochloric acid 37% (m/V) as neutralizing agent and $FFeCl_3$ > 97% were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Silica gel and aluminum oxide were supplied by Fisher Scientific GmbH (Schwerte, Germany). *S*- and racemic ibuprofen were kindly provided by Pen Tsao chemical industry ltd. (Hong Kong, China). Ultrapure water for CE measurements was produced by a water purification system from Merck Millipore (Darmstadt, Germany). 0.20 μm PVDF filters were supplied by Carl Roth GmbH (Karlsruhe, Germany). For filtration of reaction mixtures, Labsolute filter circles type 2020 DIN 53 137 (5–13 μm) from Th. Geyer GmbH & Co.KG (Renningen, Germany) were used.

2.2 Instrumentation

Stressing experiments (20–30 Hz milling frequencies) were performed using a Retsch MM400 ball mill from Retsch GmbH (Haan, Germany) with a milling jar (10 mL) and milling ball (10 mm diameter) made of ZrO_2-Y (zirconia dioxide stabilized with yttria) or stainless steel. An IST 636

ball mill from InSolido Technologies (Zagreb, Croatia) was used for the milling frequency of 35 Hz. For evaporation of solvents, a Heidolph Laborota 4000 rotary evaporator from Heidolph Instruments GmbH & Co.KG (Schwabach, Germany) was used.

CE separations were performed using a P/ACE MDQ capillary electrophoresis system from Beckman Coulter (Krefeld, Germany), equipped with a DAD. The fused silica capillaries purchased from BGB Analytik (Rheinfelden, Germany) were of 60.2 and 50.2 cm total length (50.0 or 40.0 cm effective length) with an i.d. of 75 and 50 μm, respectively.

2.3 CE procedure

The final method was carried out on a 60.2 cm (50.0 cm effective length) x 75 μm fused silica capillary. The optimized BGE (background electrolyte) consisted of 35 mM TM-β-CD, 50 mM sodium acetate buffer (pH 5.0), and 10 mM magnesium acetate tetrahydrate. The cartridge temperature was maintained at 27°C and a voltage of + 26 kV was applied ($E = 432$ V/cm, current approximately 75 μA). The detection was performed using a DAD at $\lambda = 202$ nm (high sensitivity filter). The samples were injected hydrodynamically by applying a pressure of 3.45 kPa for 5 s on the anode side. Sample concentration was 0.6 mg/mL in 0.005 M NaOH.

New capillaries were conditioned by successively rinsing with water (1 min), 1 M NaOH (10 min), water (1 min), 2 M HCl (10 min), water (1 min) and BGE (5 min), followed by an equilibration step with the BGE at 20 kV for 30 min. Before daily measurements, the capillary was successively flushed with 1 M NaOH (3 min), 0.1 M NaOH (3 min), water (1 min), and BGE (5 min). In between measurements, the capillary was flushed with water (0.5 min), 0.1 M NaOH (6 min), water (1 min), and BGE (3 min) directly before injection. The applied pressure during the rinsing steps was 138 kPa. For separation sequences, each buffer vial was used once only.

2.4 Stressing of S-Ibu

Isomerization tests were performed using *S*-Ibu in form of its free acid according to the milling processes shown in Tables S1 and S2 ($n = 1$, Supporting Information). For each experiment, the milling jar and milling ball were of the same material: ZrO_2-Y for schemes **a**, **b**,**d**–**g** or stainless steel for scheme **c**. For each milling process, the jar was loaded with 103.1 mg of *S*-Ibu (0.5 mmol) and optionally additives before milling. KOH was used as a basic additive at 2.0 molar equivalents (56 mg), and $FeCl_3$ as a Lewis acid at 0.1 molar equivalents (8.0 mg), respectively. As grinding auxiliaries, 103 mg of SiO_2 (1 weight equivalent) or 500 mg Al_2O_3 were applied.

In case of using KOH as an additive, after the milling procedure, the mixture was neutralized with an aqueous solution of 1 M HCl. The pH was checked to be 6–7, using pH paper (schemes **b**, **f**, **g**). Afterward, the mixture was extracted with ethyl acetate and evaporated. If grinding auxiliaries such as SiO_2 or Al_2O_3 were applied, the extracted mixture was filtered before evaporation. The resulting powder was used for CE analysis.

2.5 Preparation of solutions for CE

2.5.1 BGE solutions

1 M sodium acetate buffer was prepared by dissolving 1.43 mL of acetic acid ≥ 99.8% in water, adjusting to the desired pH with 10 M sodium hydroxide solution and subsequent dilution to 25.0 mL with water. The 0.1 M magnesium acetate solution was prepared by dissolving 536 mg of magnesium acetate tetrahydrate in water and diluted to 25.0 mL with water. BGE stock solutions were stored at 4°C, protected from light and used within 7 days. The final BGE was always freshly prepared by mixing the appropriate volumes of acetate buffer and magnesium acetate stock solutions, adding the required amount of the respective CD. The mixture was diluted with water to achieve the desired concentration.

2.5.2 Ibu solutions

Stock solutions of *S*- and racemic Ibu were prepared by dissolving 50.0 mg of the respective compound in 10.0 mL aqueous 0.05 M NaOH.

Test solutions for validation were prepared by mixing 600 μL of *S*-Ibu stock solution with varying amounts of rac-Ibu stock solution, diluting with water to 5.0 mL. For initial method development, rac-Ibu solutions were prepared by diluting a 5 mg/mL stock solution of rac-Ibu in methanol to a concentration of 0.4 mM. For the method optimization on the larger i.d. capillary, a 0.6 mg/mL *S*-Ibu solution in 0.005 M NaOH was used. All solutions were stored at 4°C, protected from light.

The sample solutions were prepared by dissolving the respective sample in 0.005 M NaOH, resulting in a concentration of 0.6 mg/mL. Samples were vortexed and sonicated until complete dissolution. Sample measurement was conducted in randomized order in triplicates. The solutions were passed through a 0.20 μm PVDF filter and degassed prior to injection.

3 Results and discussion

3.1 Method development

According to Rawjee et al. [24], only the uncharged forms of the Ibu enantiomers are interacting with the CD as chiral selector. However, when using a neutral CD, differences in electrophoretic mobility and hence resolution are only possible if Ibu ($pK_S \approx 4.5$ [43]) is partially charged. Consequently, the buffer pH plays an important role for the resolution of the Ibu enantiomers.

Table 1. Buffer and pH testing results

pH	Acetate (mM)	t_m *S*-Ibu	R_S	Current (μA)
4.5	25	6.40	n.a.	9
	50	8.23	n.a.	20
	75	9.35	n.a.	29
5	25	6.89	n.a.	16
	50	9.16	n.a.	36
	75	11.05	n.a.	52
	100	10.48	1.05	69
	125	12.08	1.32	81
5.5	25	7.29	n.a.	20
	50	9.63	n.a.	45
	75	12.20	1.33	65
	100	11.95	1.37	78
	125	12.91	1.62	95
	150	15.85	1.86	123
5.7	150	19.80	2.02	138
	175	18.61	1.86	143

n.a. = not available.

The abovementioned concept was adopted for the CE method development approach, using an untreated fused silica capillary and applying the normal polarity mode (anode at the inlet). TM-β-CD was used as chiral selector because it has been proven to be well suitable for chiral recognition of the Ibu enantiomers [26]. An acetate buffer was chosen because it is suitable for ensuring a stable pH near the pK_S of Ibu. The separation was initially developed on a 50.2 cm x 50 μm fused silica capillary, investigating the effects of the pH, the concentration of BGE components (buffering agent, cationic additives) and TM-β-CD on the migration time and resolution R_S of the Ibu enantiomers. R_S was calculated according to the equation

$$R_S = 1.18 \times (t_{m1} - t_{m2}) / (w_{0.5(1)} + w_{0.5(2)}) \quad (1)$$

where t_m is the migration time and $w_{0.5}$ is the peak width at half height. To enhance sensitivity, the method was transferred to a 75 μm i.d. capillary, considering the adjustment of the separation parameters.

3.1.1 Initial method development: 50 μm i.d.

The impacts of buffer concentration and pH on enantioseparation were studied on the 50.2 cm x 50 μm fused silica capillary, applying a voltage of + 20 kV. A solution of 0.4 mM racemic Ibu was used. To this end, 30 mM TM-β-CD were dissolved in different amounts of 1 M sodium acetate buffer of pH 4.5, 5.0, 5.5, and 5.7, respectively, then diluted with water to 1.0, 2.0, or 5.0 mL, depending on the volume needed. The resulting acetate concentrations were 25–175 mM. The results are summarized in Table 1. Ibu enantiomers could not be resolved at pH 4.5 and 5 employing buffer concentrations of 25–75 mM. R_S values ≥ 1.5 within about 20 min could be obtained at pH 5.5 or 5.7 with an acetate concentration of at least 125 mM. However, the resulting high currents and Joule heating led to massive band broadening, drifting, and noisy baselines.

Table 2. Results of Zn^{2+} concentration testing (1–10 mM)

Zn^{2+} (mM)	t_m *S*-Ibu	R_S	Current (μA)
1	11.28	1.37	50
2.5	11.67	1.55	48
5	13.72	1.95	54
7.5	15.28	2.19	55
10	16.10	2.33	55

Next, it was aimed to combine low separation currents and sufficient enantioresolution. The experiments were carried out using a BGE composed of 75 mM acetate buffer (pH 5.5) and 30 mM TM-β-CD, applying a voltage of 20 kV (E = 398 V/cm) and adding varying amounts of Zn^{2+} ions. Cationic additives are known to modulate the EOF by interacting with the negatively charged capillary wall. To decrease the conductivity of the BGE, zinc sulfate heptahydrate was considered as polycationic buffer additive. The Zn^{2+} concentration was varied from 1–10 mM and the effects on migration time, resolution, and current were investigated (Table 2). Migration times and resolution of the Ibu enantiomers consistently increased with rising Zn^{2+} concentration, while the resulting current did not change considerably. Thus, it can be concluded that adding Zn^{2+} ions to the BGE is more efficient than high acetate concentrations regarding resolution and separation current. However, the Zn^{2+} cations were reduced to Zn^0 on the cathode during separation, leading to arbitrary current distortions. Hence, cations with a smaller redox potential but similar size were considered, such as Mg^{2+}. 10 mM zinc sulfate heptahydrate could be replaced by the same molar amount of magnesium sulfate heptahydrate, achieving similar separation results. The separation conditions with a BGE composed of 100 mM acetate buffer pH 5.25, 15 mM $MgSO_4 \times 7H_2O$, applying a voltage of 29 kV resulted in migration times of less than 10 min for the Ibu isomers and an R_s value of 2.1.

The TM-β-CD concentration was varied between 10 and 80 mM to study the impact on migration time, resolution, and current. The respective results are depicted in Fig. 2. Higher TM-β-CD concentrations increased the migration times and the resolution of the Ibu enantiomers (Fig. 2A). The current during separation decreased due to the lowering conductivity of the BGE (Fig. 2B). In fact, satisfying resolution ($R_S \geq 2$) and migration times could be achieved with TM-β-CD concentrations of 30–40 mM.

A detection wavelength of λ = 202 nm was chosen, even though the Ph.Eur. used λ = 214 nm in the HPLC method for the test on related substances [44], our CE method showed the highest sensitivity for detection of the *R*-isomer at λ = 202 nm.

To sum up the method development results obtained on the 50.2 cm x 50 μm capillary, sufficient separation ($R_S = 2.1$) could be achieved in less than 10 min using the abovementioned separation conditions. Current during separation was

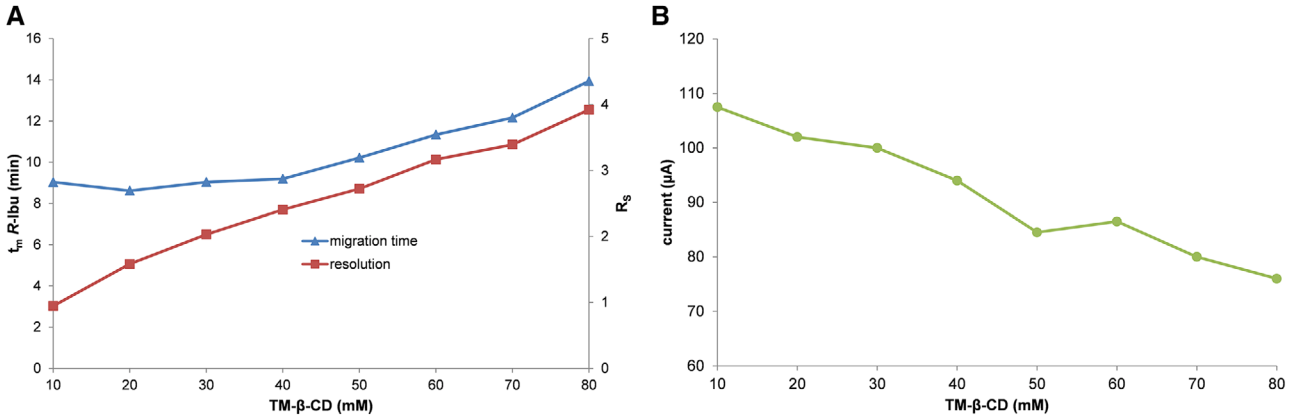

Figure 2. Results from TM-β-CD concentration testing (10–80 mM). BGE: 100 mM, sodium acetate pH 5.25, 15 mM magnesium sulfate heptahydrate. Applied voltage: + 29 kV. (A) migration times and resolution, (B) current during separation.

Table 3. Test results of methanol and acetonitrile as BGE additives (1 and 5% V/V)

	t_m *S*-Ibu (min)	R_S	Current (µA)
no organic solvent	16.23	2.39	107
MeOH (% V/V)			
1	16.41	2.11	99
5	17.94	2.17	93
ACN (% V/V)			
1	16.22	1.94	104
5	16.89	1.85	105

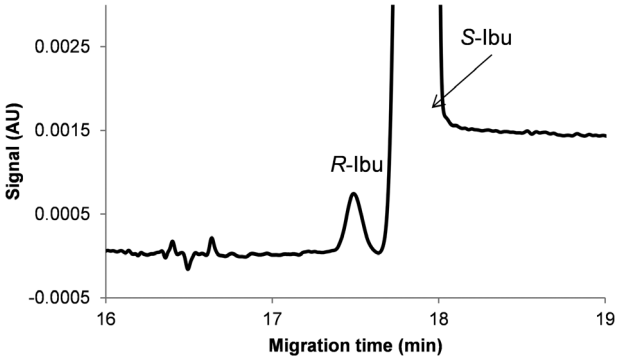

Figure 3. Electropherogram of *S*-Ibu using 5% methanol (V/V) as BGE additive.

about 90 µA (E = 578 V/cm), the resulting LOQ was calculated to be 0.53% *R*- in *S*-Ibu. To further lower the LOQ, a capillary with a larger i.d. (75 µm) was used.

3.1.2 Method transfer and optimization: 75 µm i.d.

The method was transferred to a 50.2 cm × 75 µm capillary first (40.0 cm effective length) by reducing the voltage to 12 kV leaving the BGE unchanged, resulting in a current of about 70 µA. The long migration times of more than 30 min were reduced to 16 min by adjustment of the acetate concentration to 75 mM and $MgSO_4$ x 7 H_2O to 15 mM, applying a voltage of 20 kV (current about 107 µA). Further studies involved the testing of organic solvents as buffer additives.

Organic solvents, e.g., methanol or acetonitrile, may affect the selectivity in CE enantioseparation of chiral drugs if added to the BGE [25,26,45,46]. As part of our method development, this was studied by adding 1 and 5% (V/V) methanol and acetonitrile to the BGE, respectively. As shown in Table 3, increasing amounts of the organic modifier resulted in prolonged migration times, which was more pronounced in the case of methanol compared to acetonitrile. The current during separation was slightly decreased. However, severe and irreproducible baseline shifts were observed at methanol concentrations of 5% V/V (Fig. 3). Hence, methanol and acetonitrile were withdrawn for further method development. Moreover, organic solvents were avoided for the preparation of Ibu samples because they led to noisy baselines and sample overloading effects. 0.005 M NaOH was used instead.

The cationic additives magnesium, sodium and potassium salts were tested at a concentration of 12 mM and compared with respect to separation performance. Therefore, 12 mM magnesium sulfate, magnesium chloride, magnesium acetate, calcium chloride, sodium chloride or potassium chloride were added each to the BGE. The enantiomers were not resolved using NaCl or KCl. Best results regarding resolution and migration time were achieved using magnesium acetate and magnesium sulfate. Since magnesium sulfate led to noisy baselines after the *S*-Ibu peak, magnesium acetate was applied (Table 4).

Finally, a sufficient separation (migration time about 21 min, R_S = 2.6) including a smooth baseline was achieved on a 60.2 cm (50.0 cm effective length) x 75 µm capillary after initial adjustment of the BGE components to 65 mM acetate pH 5.0, 12 mM $MgSO_4$ x 7 H_2O and 35 mM TM-β-CD, applying a voltage of 20 kV (current about 70 µA).

The capillary temperature was studied as described in Table 5. Elevated temperatures provided faster migration times

Table 4. Testing of different cationic additives

Additive	t_m *S*-Ibu (min)	R_S	Current (μA)
$CaCl_2 \times 6\ H_2O$	21.14	2.58	69
$MgCl_2 \times 6\ H_2O$	19.06	2.19	77
$MgSO_4 \times 7\ H_2O$	17.05	2.08	83
$MgAc_2 \times 4\ H_2O$	15.14	2.15	91
NaCl	13.09	n.a.	60
KCl	13.86	n.a.	64

Table 5. Testing of capillary temperature

Temperature (°C)	t_m *S*-Ibu (min)	R_S	Current (μA)
25	21.14	2.58	69
30	19.06	2.19	77
35	17.05	2.08	83
40	15.14	2.15	91

and still sufficient resolution. Best results were achieved at a temperature of 27°C.

To ensure constant migration times, the rinsing procedure between each run was optimized. Rinsing schemes were tested as suggested by Wahl and Holzgrabe [47, 48], where acidic, basic and organic liquids were used in varying orders upon flushing of a 60.2 cm (50.0 cm effective length) × 50 μm capillary. For our tests, the rinsing durations were adjusted accordingly. If no 0.1 M NaOH was applied as a rinsing solvent, the migration times dramatically increased, which was also observed when successively water, 0.1 M NaOH and BGE were applied. Flushing with organic solvents such as methanol followed by basic rinsing led to decreasing migration times. The optimized rinsing sequence was water (0.5 min), 0.1 M NaOH (6 min), water (1 min) and BGE (3 min). After each separation, a vial with fresh BGE was used, coping with changes such as degradation and/or electrolysis in BGE and, consequently, in separation conditions.

Injection parameters including injection duration and sample concentration were optimized to achieve the maximum sensitivity, which was determined by evaluation of the *R*-Ibu peak height. The injected *S*-Ibu concentration was tested from 0.1 to 0.8 mg/mL, applying a pressure of 3.45 kPa for 4 s at the anode side. As shown in Fig. 4, the peak height got more intense with increasing *S*-Ibu concentration, reaching a maximum at about 0.65 mg/mL. At higher concentrations, the peak height dropped because of peak broadening due to overloading effects. The resolution decreased because of the broadening Ibu peaks, but was sufficient throughout the tested concentration range ($R_S > 2$). Along these lines, long injection times also resulted in a decrease of resolution and increase of the peak height (Fig. 5).

The resolution decreased because of the broadening Ibu peaks, but was sufficient throughout the tested concentration range ($R_S > 2$) The *S*-Ibu peak area increased linear with the tested concentration until 0.65 mg/mL, which may be indicative for beginning sample overloading at higher concentrations. Thus, preserving a safety margin, a slightly decreased *S*-Ibu concentration of 0.6 mg/mL was used. The injection duration was varied from 3 to 10 s at a pressure of 3.45 kPa. Figure 5 shows a linear increase of the *R*-Ibu peak height from 4 to 10 s, but also a severe decrease of resolution at injection durations above 5 s. As a compromise the following injection parameters were chosen: a S-Ibu concentration of 0.6 mg/mL, which were injected for 5 sec at a pressure of 3.45 kPa.

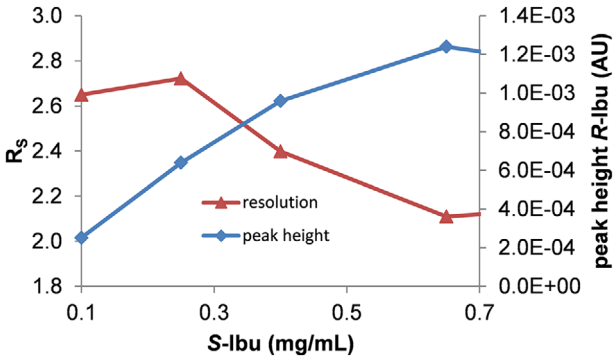

Figure 4. Resolution and *R*-Ibu peak height as a function of the *S*-Ibu concentration (mg/mL).

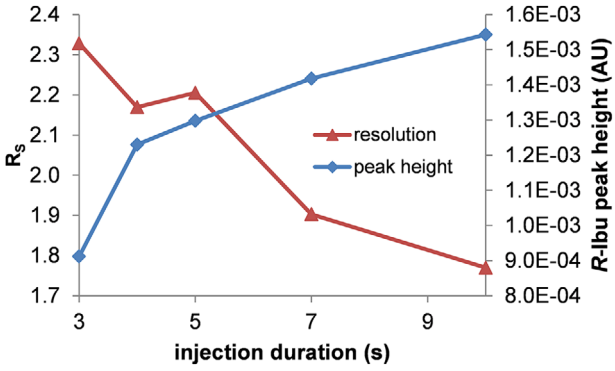

Figure 5. Resolution and *R*-Ibu peak height as a function of the injection duration (s).

An electropherogram of *S*-Ibu (0.6 mg/mL) containing 0.68% *R*-Ibu is shown in Fig. 6. Good separation of the enantiomers ($R_S \geq 2.3$) could be achieved within about 18 min with the minor enantiomer (*R*) migrating in front of (*S*). Quantification of *R*-Ibu was performed by area normalization using the migration time corrected area A_{corr} (area divided through migration time of the peak). The percentage content of *R*-Ibu was determined using

$$\left(A_{corr}\ (\mathrm{R-Ibu}) / \sum A_{corr}\right) \times 100. \quad (2)$$

The sample of *S*-Ibu provided by Pen Tsao contained a small amount of the *R*-isomer (0.68%), which can be regarded as an advantage, because it is not necessary to achieve an LOQ below 0.1% for *R*-Ibu as generally recommended by the Ph.Eur. for impurities.

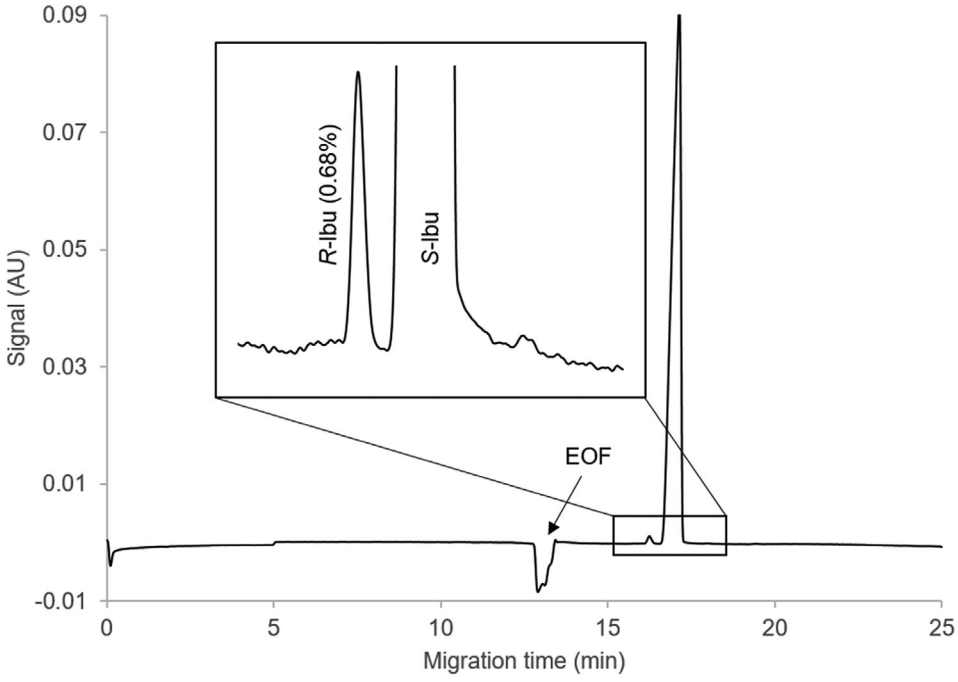

Figure 6. Electropherogram of the *S*-Ibu sample (0.6 mg/mL). Electrophoretic conditions: BGE: 35 mM, TM-β-CD, 50 mM, sodium acetate pH 5.0, 10 mM, magnesium acetate tetrahydrate, fused silica capillary, untreated 60.2 cm (50.0 cm from inlet to detector) × 75 μm i.d.; voltage: + 26 kV; temperature: 27°C; detection: DAD with λ = 202 nm; injection, 5.0 s at 3.45 kPa; current: 75 μA.

3.2 Method validation

The validation was performed according to the ICH Guideline Q2(R1) [49]. All measurements were conducted in randomized order.

The linearity was determined at five concentration levels ranging from 0.68 to 5.49% *R*- in *S*-Ibu ($y = 160.09x + 0.0705$). Each concentration level was measured in triplicate. The coefficient of determination was found to be ($R^2 = 0.9991$). Accuracy was evaluated by determining the recovery ranging from 97 to 103% (Table S3). The small amount of *R*- in *S*-Ibu (0.68%) was taken into account by subtracting the area of the *R*-Ibu peak from the area of the *S*-Ibu peak.

The LOQ of *R*-Ibu in the presence of *S*-Ibu was determined based on an S/N of 10 by plotting the percentage *R*-Ibu against the corresponding S/N. As already mentioned, the pure *S*-Ibu sample contained 0.68% *R*-Ibu; thus the LOQ was determined by extrapolation of the calibration curve ($y = 108.59x - 12.625$; $R^2 = 0.9984$). The extrapolated LOQ was found to be 0.21% for *R*-Ibu, referring to a concentration of 1.25 μg/mL. A second approach for evaluating the LOQ of *R*-Ibu was performed by dilution of a solution of rac Ibu until an S/N of 10 was reached and determination of the corresponding concentration of *R*-Ibu. The results are summarized in Table S4. The slight difference in determined LOQs pointed out in percentage *R*-Ibu (0.21 and 0.15%, respectively) may be explained by the presence of massive excess of *S*-Ibu. The comparison of the LOQs of other CE methods for the evaluation of the enantiomeric purity of *S*-Ibu (1% [37] and 0.5% [39]), revealed our method to be superior with regard to sensitivity (0.21%).

Evaluation of precision comprises repeatability and intermediate precision. The repeatability was determined by the measurement of a solution of *S*-Ibu (0.6 mg/mL) containing 0.68% R-Ibu ($n = 6$): an RSD of 1.52% for the *R*-Ibu content was found (Table S5). The RSD of the migration times was 1.31% for the *R*-Ibu and 1.46% for the *S*-Ibu peak. For intermediate precision (consecutive measurements on 2 days), with freshly prepared sample and BGE stock solutions, the RSD of the percentage *R*-Ibu content was 1.97% and of the migration times for the *R*-Ibu and the *S*-Ibu peak 2.65% and 2.78%, respectively, indicating a sufficient precision of *R*-Ibu determination.

3.3 Measurement of stressed *S*-Ibu samples

Figures 7A and 7B show the CE results of isomerization after applying the milling schemes **a**–**g**. As can be seen, without the addition of KOH neither increasing the milling speed nor the reaction time leads to an isomerization of *S*-Ibu (Fig. 7A). However, a partial isomerization was observed when KOH was used. *R*-Ibu increased significantly when a combination of high milling frequency and extended milling time was applied. In particular, after milling for at least 30 h at 20 Hz milling frequency, a significant isomerization was observed (samples 43, 45, and 46, Fig. 7A). Only at a milling frequency of 25 Hz or higher an isomerization was already detected after 2 h of milling. To observe isomerization at a milling time of 20 min, a milling frequency of 35 Hz had to be applied (sample 39). Certain samples from milling schemes **a** and **b** were stored in solution at 4°C for 7 days to study further isomerization (samples 21, 23, 39, 43, 44, 46). No increase in

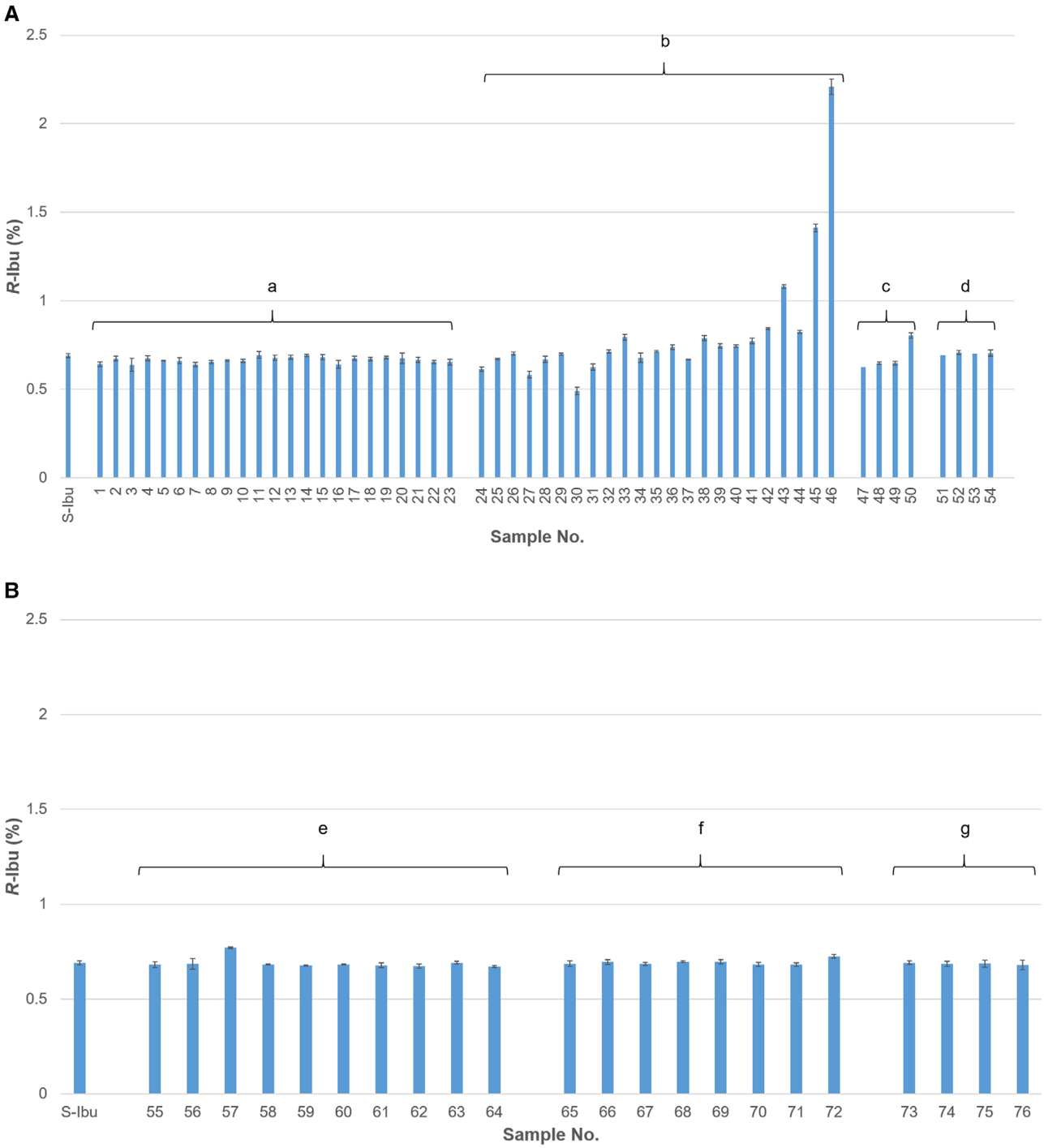

Figure 7. Results from isomerization tests obtained by CE measurements ($n = 3$). (A) Samples from scheme a-d, (B) samples from scheme e-g. For sample assignment, see Tables S1 and S2

percentage *R*-Ibu could be detected, indicating an isomeric stability of *S*-Ibu in solution for at least 7 days.

Under acidic conditions, a slight isomerization was detected for sample 50 (0.80% *R*-Ibu, Fig. 7A). In contrast, sample 48 did not show any increase in the content of *R*-Ibu (0.65% R-Ibu) which indicates that the milling speed might be a crucial factor for isomerization for this milling scheme. No isomerization was observed in the case SiO_2 was used as the only grinding auxiliary (scheme **d**, Fig. 7A). For milling scheme **e**, water was applied as a liquid assisted grinding (LAG) agent [50] without further additives. As for schemes **a** and **d**, no isomerization could be ascertained, except for

sample 57 (0.77% *R*-Ibu, Fig. 7B), where 0.1% molar equivalents of water were used. However, for sample 60, where the five-fold amount of water was employed, no isomerization was detected (0.68% *R*-Ibu). Process scheme **f** combines the usage of KOH and LAG agent. Here, only sample 72 shows minor isomerization of *S*-Ibu (0.72% *R*-Ibu, Fig. 7B). Sample 33 reveals a higher percentage of *R*-Ibu (0.80%) where no water was added. Consequently, the effect of water on isomerization of *S*-Ibu could be considered negligible. The conditions used for scheme **g** did not lead to isomerization (Fig. 7B).

4 Concluding remarks

The CE method development revealed the addition of small cations such as Mg^{2+} or Zn^{2+} to highly increase the separation of Ibu enantiomers. The developed and validated CE method is proven to be capable to reliably determine the enantiomeric purity of *S*-Ibu.

To our knowledge, the isomerization of *S*-Ibu stressed in a ball mill has not been described before. The results extend the knowledge of *S*-Ibu isomerization under mechanical stress and several comparatively mild stressing conditions. Measurement results indicate that significant isomerization of *S*-Ibu only occurs on milling in presence of KOH, which gets more intense when milling time and milling speed exceed particular values. However, the extent of isomerization is limited as shown by the small increase of percentage *R*-Ibu, which emphasizes the stereochemical stability of *S*-Ibu under the conditions tested. Since a slight isomerization was only observed under basic conditions, which do not occur in tablets, capsules, and ointments, formulations are most likely stable upon storage.

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Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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