APPLICATION OF THIN-LAYER CHROMATOGRAPHY (TLC) TO INVESTIGATING OSCILLATORY INSTABILITY OF THE SELECTED PROFEN ENANTIOMERS

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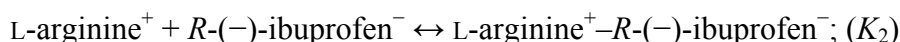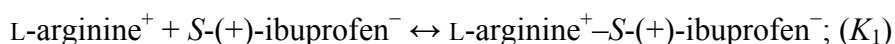
SUMMARY

In our earlier studies we intended to evaluate the performance of thin-layer chromatography (TLC) as an analytical tool applied to separation of the selected profen (i.e. 2-arylpropionic acid) enantiomers and we selected ibuprofen as a model analyte for this purpose. Surprisingly enough, TLC is relatively seldom used for separation of enantiomers, even though – despite the low number of theoretical plates (N) in this particular technique in comparison with fully automated column chromatographic techniques (basically GC and HPLC) – it seems very well suited for complete separation of two analytes only (i.e. a pair of enantiomers).

Separation of profens can be regarded as a particularly urgent analytical task, because they are among the most widely prescribed synthetic non-steroidal anti-inflammatory drugs and in medicinal chemistry we must be vigilantly aware of possible unpredictable consequences of dispensing medicines as racemic mixtures.

In earlier and in more recent studies we have provided enough evidence not only to prove that the analytical performance of TLC is sufficient for separation of the enantiomers of ibuprofen, but also – even more important – to demonstrate the usefulness of this simple yet very efficient separation technique in physicochemical studies of the oscillatory instability of profens when stored for long periods of time in aqueous media. By use of TLC it was possible to show the oscillatory transenantiomerization of the profens from the *S* to the *R* form, and *vice versa*, during storage. The correctness of our TLC assessment with regard to structural oscillation of enantiomeric profens was later successfully confirmed by means of polariemetry.

INTRODUCTION

In their pioneering paper on the thin-layer chromatographic separation of ibuprofen enantiomers, Bhushan and Parshad [1] suggested silica with addition of L-arginine as stationary phase and ACN–MeOH–H$_2$O, 5:1:1 (v/v), plus several drops of acetic acid to maintain the pH < 4.8, as mobile phase. Their experimental procedure included in-house coating of glass plates with silica–gypsum slurry containing an accurately known amount of L-arginine as chiral selector. This direct approach (i.e. using a chiral stationary phase) to separation of the enantiomers of ibuprofen we later extended [2] to thin-layer chromatographic systems with commercially available silica-coated glass plates, again impregnated with L-arginine. Because mobile-phase pH must be maintained below the isoelectric point (pI) of L-arginine (pH 4.8), the most probable mechanism of separation of the two ibuprofen enantiomers can be given by the equations:

L-arginine$^+$ + S-(+)-ibuprofen$^-$ $\leftrightarrow$ L-arginine$^+$–S-(+)-ibuprofen$^-$; (K_1)

L-arginine$^+$ + R-(−)-ibuprofen$^-$ $\leftrightarrow$ L-arginine$^+$–R-(−)-ibuprofen$^-$; (K_2)

Successful separations of ibuprofen enantiomers were shown as chromatographic bands visualized by use of iodine vapour [1] and densitometrically [2]. This separation success was, however, rather limited, because storage for several hours or days of ibuprofen dissolved in EtOH–H$_2$O, 7:3 (v/v) resulted in the surprise disappearance of the two well separated chromatographic bands and, instead, the steadily changing position of a single and skewed ibuprofen band. On successive chromatograms this
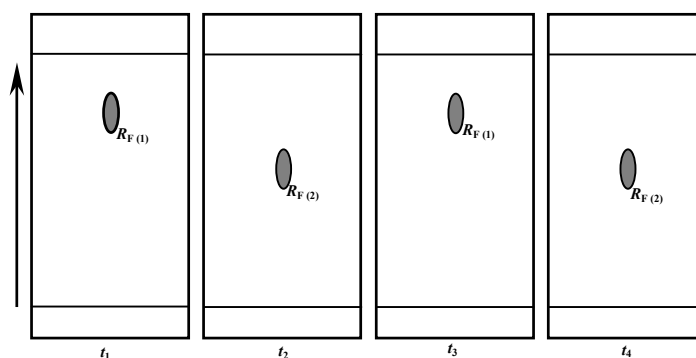

Fig. 1

Schematic representation of oscillatory changes in the R_F values of ibuprofen (and other investigated profens also) as a function of storage time in EtOH–H$_2$O, 7:3 (v/v)

single and skewed ibuprofen band appeared in quite different positions which clearly depended on the storage time yet which remained within rigid boundaries, i.e. the uppermost and the lowermost R_F values, thus giving the impression of 'oscillation'. This phenomenon is shown schematically in Fig. 1.

Because of our curiosity about the reason for this behaviour, a more systematic study of the oscillating transenantiomerization of S-(+)-ibuprofen became the target of research described in this paper. In addition to S-(+)-ibuprofen, the behaviour of two other 2-arylpropionic acids, S-(+)-naproxen and S,R-($\pm$)-2-phenylpropionic acid, was also investigated. The outcome for the three profens is presented in this paper.

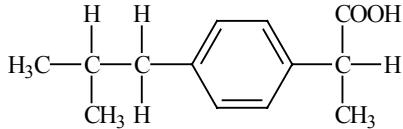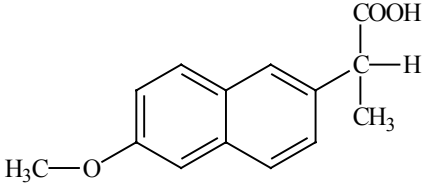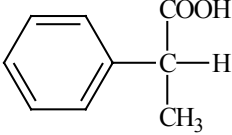## EXPERIMENTAL

Profen Samples and Standards

S-(+)-ibuprofen and S-(+)-naproxen were obtained from Sigma–Aldrich (St Louis, MO, USA; #I-106 and 28,478-5, respectively) and S,R-($\pm$)-2-phenylpropionic acid from Merck (Darmstadt, Germany; #8.20651.0010). For TLC analysis, solutions of S-(+)-ibuprofen, S-(+)-naproxen, and S,R-($\pm$)-2-phenylpropionic acid (1 µg µL^{-1}, equivalent to ca. 5.8×10^{-3}, 4.3×10^{-3}, and 6.5×10^{-3} mol L^{-1}, respectively) were prepared in 70% ethanol and 10-µL volumes were applied to the plates. For polarimetry, solutions of S-(+)-ibuprofen, S-(+)-naproxen, and S,R-($\pm$)-2-phenylpropionic acid (50, 12.5, and 500 µg µL^{-1}, respectively, equivalent to ca. 0.25, 5.4×10^{-2}, and 3.33 mol L^{-1}). The chemical structures and optical rotation properties of the profens are given in Table I.

Storage of the Test Analytes

Solutions of S-(+)-ibuprofen, S-(+)-naproxen, and S,R-($\pm$)-2-phenylpropionic acid in EtOH–H$_2$O, 7:3 (v/v) were stored at two different temperatures (6 ± 2°C and 22 ± 2°C) for prolonged periods of time (12 days for ibuprofen and 5 days with the other two profens) and R_F values were measured for each compound and each experimental series twice a day at 5-h intervals. It perhaps needs adding that for ibuprofen measurements of R_F were not performed for one day during this twelve-day period. Each storage experiment was repeated twice, thus providing the two series of data.

Table I

Schematic representation of the chemical structures of the three profens discussed in this study and the specific rotation, $[\alpha]_D$, of their S and R enantiomers at 25°C, as taken from literature

Profen	Chemical structure	Specific rotation, $[\alpha]_D$ (°)	
		S	R
Ibuprofen		+53.2 [3]	−57.5 [4]
Naproxen		+64.9 [5] +66.3 [6]	−67.2 [6]
2-Phenylpro-pionic acid		69.2 [7] 79.2 [4]	−80.0 [4]

Commercial TLC Silica Gel Layers and Their Pretreatment

TLC was performed on commercial glass plates precoated with 0.25 mm layers of silica gel 60 F_{254} (Merck, #1.05715). Before use, the plates were carefully washed by predevelopment with methanol–water, 9:1 (v/v), then dried at ambient temperature for 3 h. Washing of the plates before more 'sensitive' separations is often recommended by the manufacturer.

The washed and dried plates were then impregnated with a 3×10^{-2} mol L^{-1} solution of L-arginine in methanol by conventional dipping for 2 s. The concentration of the impregnating solution was calculated as that depositing 0.5 g L-arginine per 50 g of the dry silica gel adsorbent layer. Finally, the washed, impregnated adsorbent layers were ready for chromatography.

Development of the Chromatograms

S-(+)-Ibuprofen

Development of the ibuprofen samples was performed at two different temperatures, 6 ± 2 and $22 \pm 2°C$. Plates with three adjacent spots from the 5-µL volumes of *S*-(+)-ibuprofen solution were developed to a distance of 15 cm using the ternary mobile phase ACN–MeOH–H_2O, 5:1:1 (*v/v*) containing several drops of acetic acid to fix the pH < 4.8. After development of the chromatograms, the plates were dried at ambient temperature for 3 h and the three lanes were scanned densitometrically. The experiment was repeated twice for each measurement temperature.

S-(+)-Naproxen

Development of the naproxen samples was performed as described for ibuprofen except that the mobile phase was ACN–MeOH–H_2O, 5:1:1.5 (*v/v*), again containing several drops of acetic acid to fix the pH < 4.8.

S,R-(±)-2-Phenylpropionic acid

Development of the 2-phenylpropionic acid samples was performed as described for ibuprofen and naproxen except that the mobile phase was ACN–MeOH–H_2O, 5:1:0.75 (*v/v*), containing several drops of acetic acid to fix the pH < 4.8.

Densitometric Assessment of the Chromatograms

Densitograms were acquired with a Desaga (Heidelberg, Germany) model CD 60 densitometer equipped with Windows-compatible ProQuant software. Concentration profiles of the development lanes for the three profens were recorded in UV light from the deuterium lamp (in reflectance mode) at 210 nm. (This is the approximate wavelength of the stronger of the UV absorption maxima for ibuprofen; the stronger maxima for naproxen and 2-phenylpropionic acid are close to this position). The dimensions of the rectangular light beam were 0.02 mm × 0.4 mm. The maxima of the concentration profiles were used for calculation of R_F values.

Polarimetric Measurements of Specific Rotation $[\alpha]_D$ of the Profens

Measurements of the specific rotation $[\alpha]_D$ of solutions of *S*-(+)-ibuprofen, *S*-(+)-naproxen, and *S,R*-(±)-2-phenylpropionic acid in 70% ethanol were performed at $6 \pm 2°C$ and $22 \pm 2°C$, during storage of the solutions, by means of a Polamat A polarimeter (Carl Zeiss, Jena, Germany). The

optical path length of the measurement cell was 10 cm (= 1 dm). Specific rotation $[\alpha]_D$ was calculated by use of the standard equation $[\alpha]_D = 100\alpha/cd$, where α is the measured rotation, D denotes the wavelength employed, 589 nm (which corresponds with the sodium D line), c is the concentration of a given compound in grams per 100 cm^3 solution, and d is the measured sample thickness in decimetres (dm).

RESULTS AND DISCUSSION

Our preliminary observations of the oscillatory behaviour of ibuprofen (i.e. a regular sequential change of its steric arrangement from the *S* configuration to the *R* configuration and back) are depicted schematically in Fig. 1. These observations were made before commencement of the research discussed in this study. Even at that initial stage we managed to gather convincing enough evidence that the phenomenon observed was not because of any destruction of ibuprofen (for example decomposition, reduction etc.) but simply because of periodic alteration of its chiral configuration. Among the techniques used to gather such evidence we first used in-situ recording of the UV spectra of the bands (irrespective of R_F value) and HPLC analysis of the long-stored ibuprofen solution samples (performed in parallel with the TLC investigation).
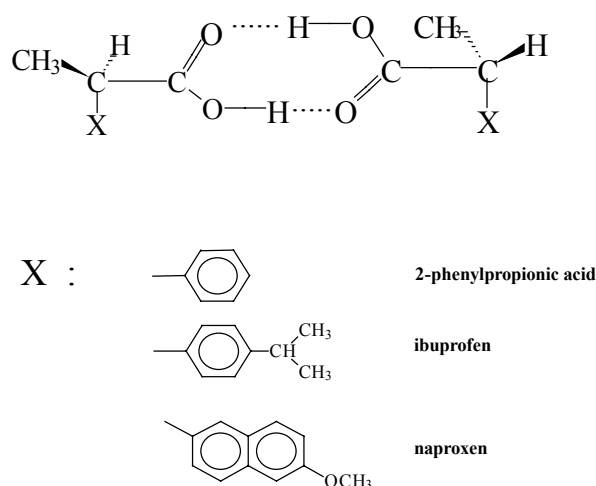

Fig. 2

The H-bonded self-association of the two enantiomeric profen molecules, resulting in a cyclic dimer

Another important preliminary observation made before this study was that after storage of a solution of S,R-($\pm$)-ibuprofen in 70% ethanol for several hours the initial separation of the two antipodes disappeared and instead a relatively broad and skewed band appeared. This could, of course, be because of the beginnings of a change in the chiral characteristics of the two enantiomers in the racemic mixture, but it is equally probable it happened because of the formation of cyclic dimers between the S and the R antipodes. As reported elsewhere [8], mixed hydrogen bonding interactions can sometimes prevent chromatographic separation of two completely different compounds; this is even more likely for two antipodes of a racemic mixture. Cyclic hydrogen-bonded dimeric structures quite typical of monocarboxylic acids and, therefore, also typical of ibuprofen, naproxen, and 2-phenylpropionic acid are shown in Fig. 2.

Now let us report the experimental results gathered in this study. As already mentioned, we started by scrutiny of the numerical values of the retardation factors (R_F) for S-(+)-ibuprofen, S-(+)-naproxen, and S,R-($\pm$)-2-phenylpropionic acid (each analyte dissolved in 70% ethanol and stored for a long time at either 6 ± 2°C or at 22 ± 2°C); the results are shown in Figs 3–5.

As mentioned in the experimental section, each experiment was repeated three times in each series and two such series were run for each profen and for each working temperature. The results originating from the same storage series were averaged; data originating from two different storage series were qualitatively almost identical. Thus for reasons of economy in this paper we present results from a single experimental series only.

Clearly evident oscillation of the respective numerical values of R_F at both measurement temperatures is strikingly apparent for all the plots in Figs 3–5. These oscillations go far beyond the experimental error inherent in thin-layer chromatography, which are generally assumed to be ± 0.02 on the unitless scale of the R_F values. Another interesting observation for all three profens is that the amplitude of these oscillations is perceptibly higher for samples stored under refrigeration than for those kept at room temperature.

In Fig. 6 we show a sequence of the changing concentration profiles accompanying 'floating' of the R_F values, as presented schematically in Fig. 1 (Fig. 6 is, in fact, meant as a sequence of the consecutive 'movie pictures'). These changes of the concentration profiles are characteristic of all three profens, although in Fig. 6 we limit ourselves – for the sake of example – to S,R-($\pm$)-2-phenylpropionic acid only. It is readily apparent

from the series shown in Fig. 6 that changes of the R_F values for S,R-($\pm$)-2-phenylpropionic acid are accompanied by densitometrically acquired and clearly evident changes of the concentration profiles of this species. Envelopes of the examined concentration profiles shift from one extreme position to the other, convincingly mirroring steric transformation of the analyte from one enantiomeric configuration to another.
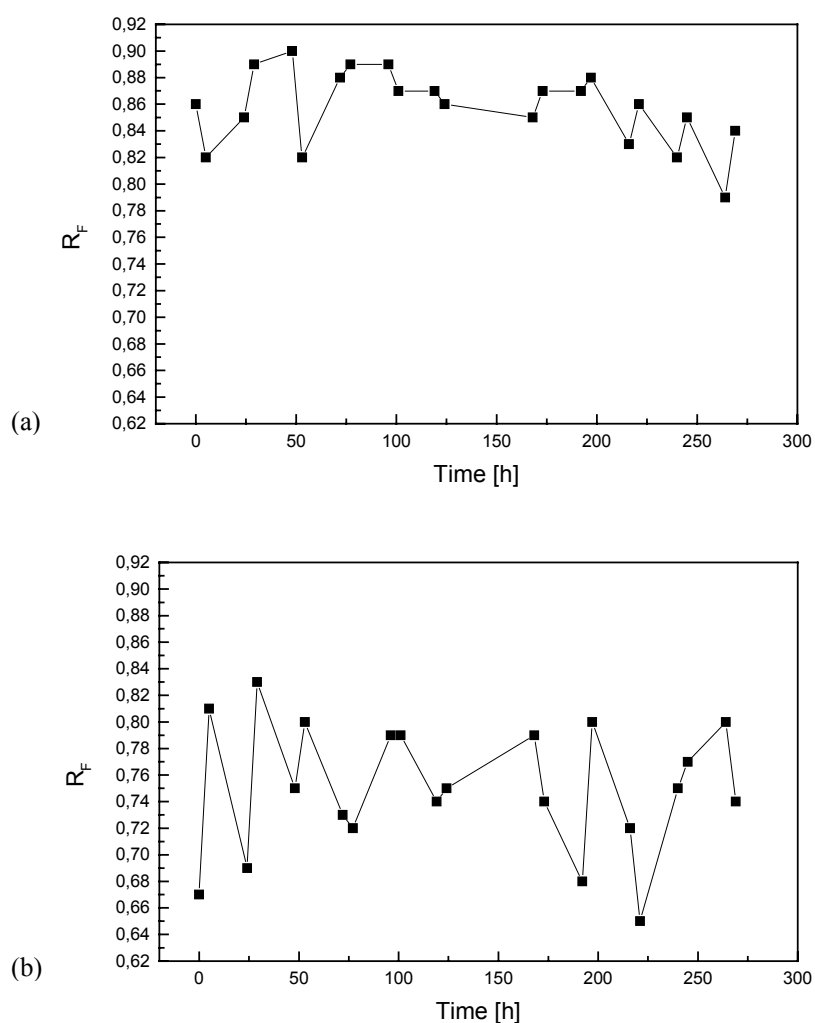

(a)

(b)

Fig. 3

Dependence of retention, R_F, for S-ibuprofen on sample storage time ($R_F = f(t)$) (a) at ambient temperature ($22 \pm 2°C$) and (b) under refrigeration ($6 \pm 2°C$)

(a)

(b)
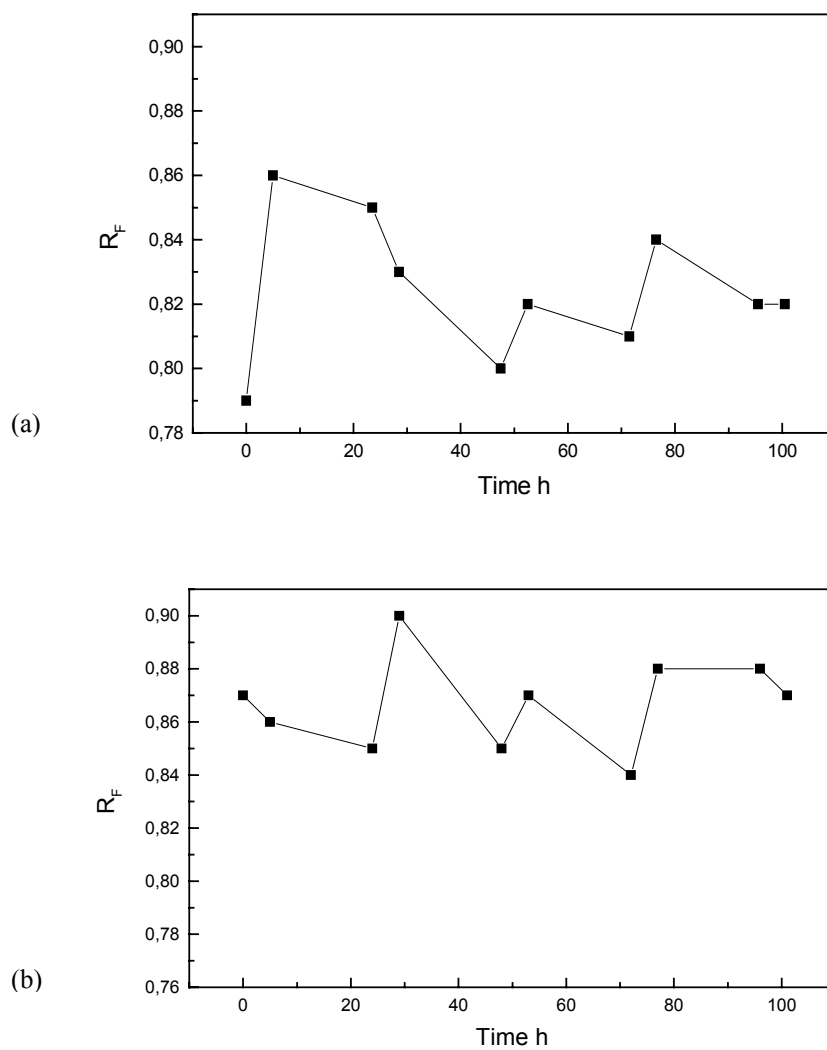
Fig. 4

Dependence of retention, R_F, for *S*-naproxen on sample storage time ($R_F = f(t)$) (a) at ambient temperature ($22 \pm 2°C$) and (b) under refrigeration ($6 \pm 2°C$)
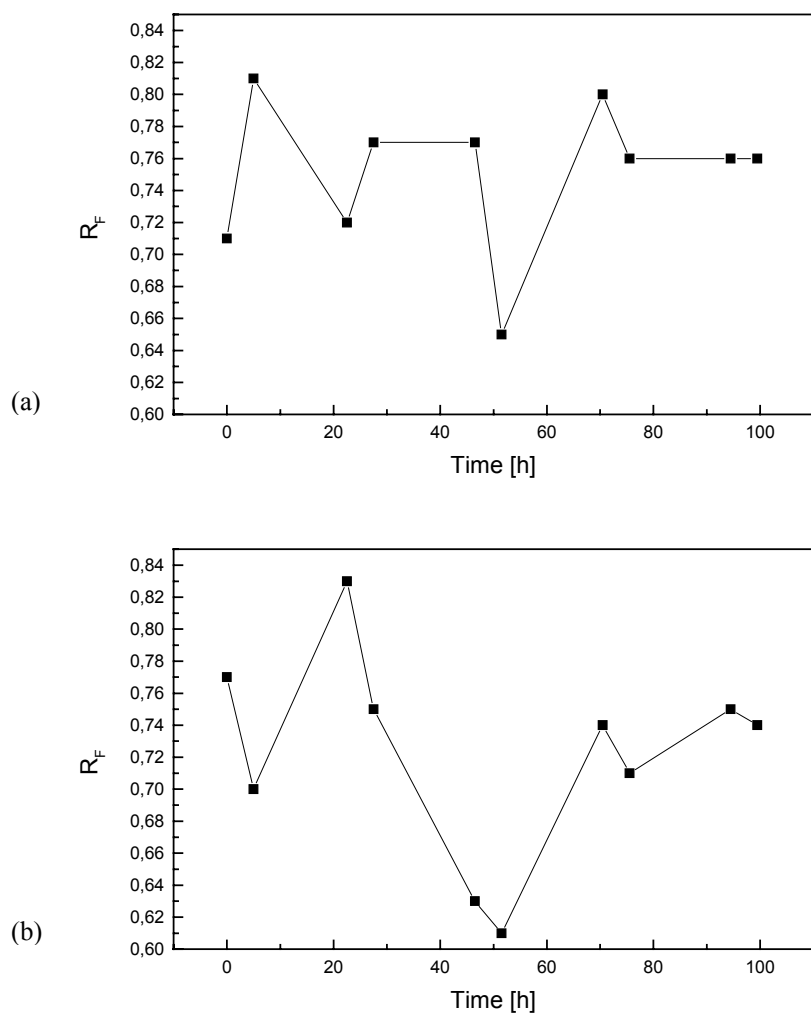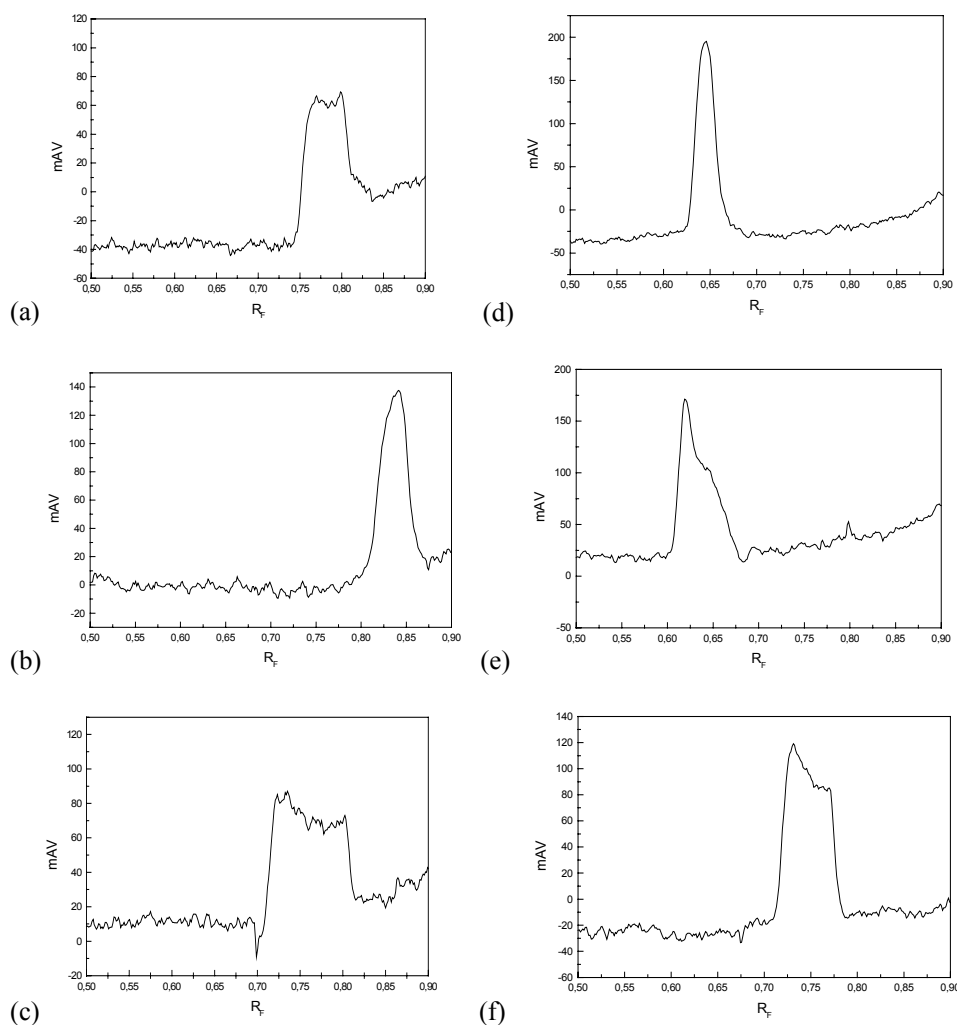
(a)

(b)

Fig. 5

Dependence of retention, R_F, for *S,R*-(±)-2-phenylpropionic acid on sample storage time
($R_F = f(t)$) (a) at ambient temperature (22 ± 2°C) and (b) under refrigeration (6 ± 2°C)

Fig. 6

Sequence of densitometric concentration profiles for 2-phenylpropionic acid after (a) 0 h (racemic mixture); (b) 22.5 h (*S*-(+) form); (c) 27.5 h (racemic mixture); (d) 46.5 h (*R*-(−) form); (e) 51.5 h (shift from *R*-(−) form to racemic mixture); and (f) 70.5 h (racemic mixture) during storage under refrigeration (6 ± 2°C). Changes of the peak concentration profiles are accompanied by changing R_F values

The thin-layer chromatographic results are good evidence of the phenomenon of oscillatory transition from one enantiomeric form of each given profen to the opposite form. Polarimetric measurements were performed to prove this hypothesis experimentally. We measured specific ro-

tation $[\alpha]_D$ of each individual profen discussed in this study during storage at $6 \pm 2°C$ and $22 \pm 2°C$. The results obtained are given in Figs 7–9.
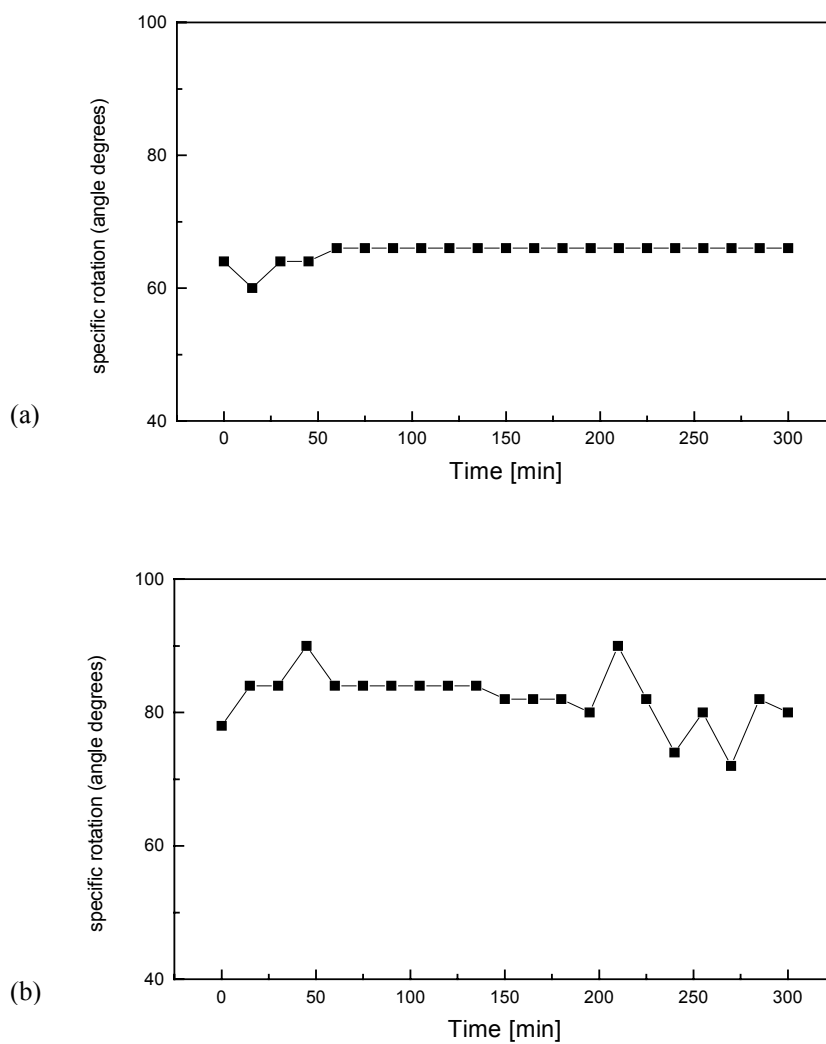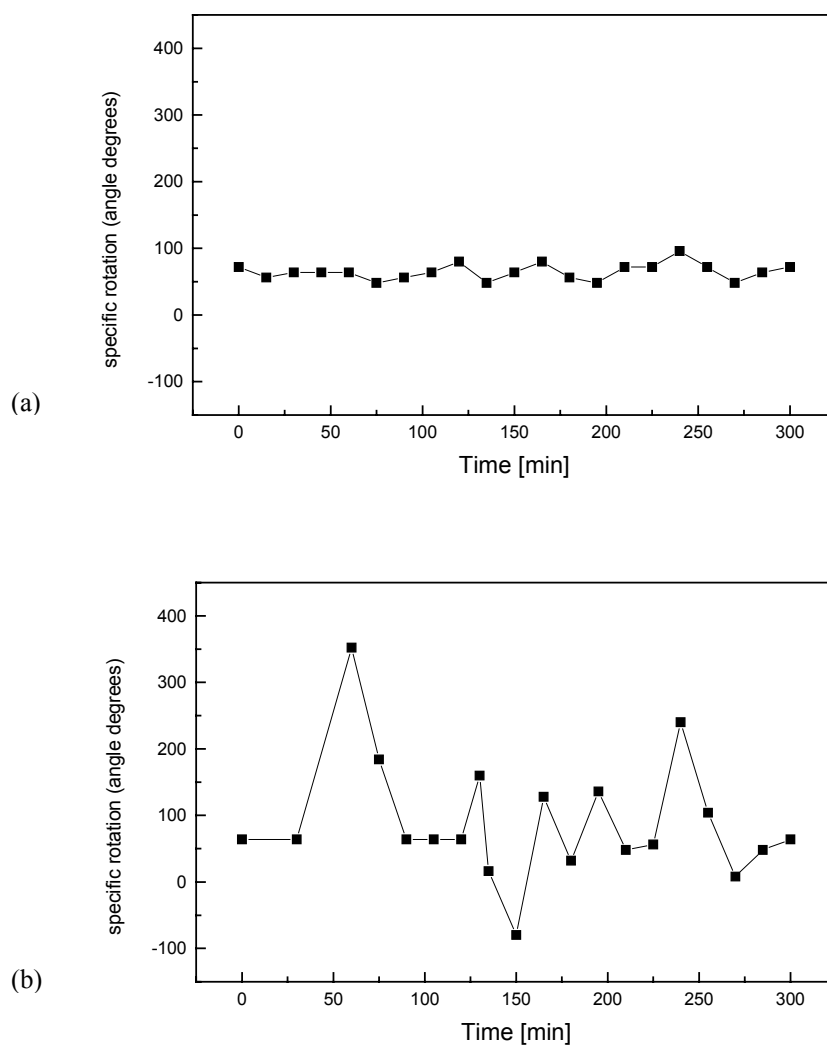

(a)

(b)

Fig. 7

Dependence of specific rotation, $[\alpha]_D$, of *S*-ibuprofen on sample storage time ($[\alpha]_D = f(t)$) (a) at ambient temperature ($22 \pm 2°C$) and (b) under refrigeration ($6 \pm 2°C$)

(a)

(b)

Fig. 8

Dependence of specific rotation, $[\alpha]_D$, of *S*-naproxen on sample storage time ($[\alpha]_D = f(t)$)
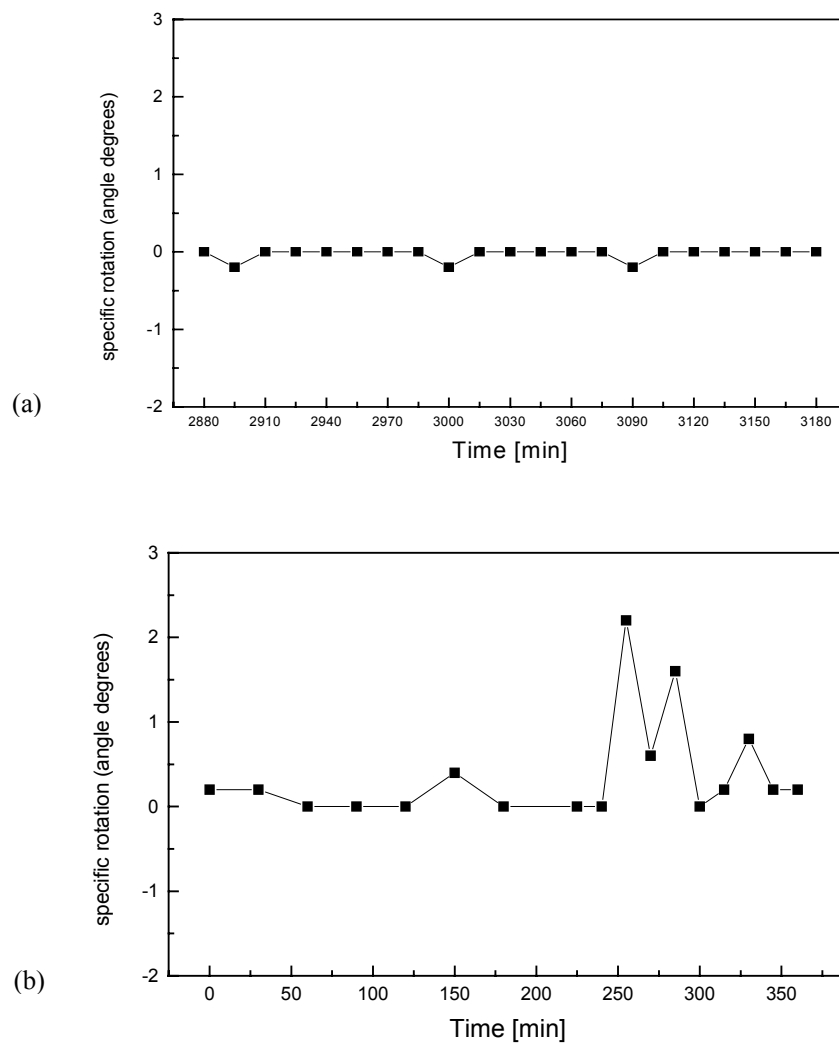(a) at ambient temperature ($22 \pm 2°C$) and (b) under refrigeration ($6 \pm 2°C$)

(a)

(b)

Fig. 9

Dependence of specific rotation, $[\alpha]_D$, of *S,R*-(±)-2-phenylpropionic acid on sample storage time ($[\alpha]_D = f(t)$) (a) at ambient temperature (22 ± 2°C) and (b) under refrigeration (6 ± 2°C)

It is apparent from these results that the measurement periods of our polarimetric studies embraced 5 h only, i.e. were much shorter than the sample storage periods covered by our TLC experiments. This is because

polarimetric measurements are incomparably faster than the thin-layer chromatographic measurements and, moreover, can be performed almost continuously (or with very short time intervals) whereas collecting a single R_F takes approximately 1 h. Nevertheless, from the plots presented in Figs 7–9 it is clearly apparent that:

  i. the specific rotation of the three investigated profens changes in an oscillatory manner in the course of time, and
  ii. the amplitude of these oscillatory changes is visibly higher at 6°C, than at 22°C (an analogous observation was valid for oscillatory changes of R_F values also).

Last, but not least, these oscillatory changes can be observed not only for the optically pure enantiomers (i.e. S-(+)-ibuprofen and S-(+)-naproxen), but also with the racemic S,R-(±)-2-phenylpropionic acid sample, although the observed amplitude of changes of optical rotation is incomparably greater with S-(+)-ibuprofen and S-(+)-naproxen, i.e. with the pure enantiomers, than with the S,R-(±)-2-phenylpropionic acid. At 6°C this amplitude ranges roughly from +70 to +90° for S-(+)-ibuprofen, and from −70° to 350° for S-(+)-naproxen whereas for S,R-(±)-2-phenylpropionic acid the amplitude ranges from 0° to slightly above +2° only. Thus these direct polarimetric results seem sufficient evidence of steady transition of one enantiomeric structure of a given antipode into that of its mirror image for all three of the profens examined in our study.

Our next step was to reflect on the molecular mechanism of this transenantiomerization of profens. In the literature [9] we found a report of a proved (and base-catalyzed) ibuprofen racemization mechanism occurring as a result of keto–enol tautomerism. From a general knowledge of keto–enol tautomerism we also know that this process can also be acid-catalyzed, and all the profens are carboxylic acids with relatively well pronounced electrolytic dissociation. Thus it seems fully justified to expect keto–enol transenantiomerization of profens in aqueous media because of the self-catalytic effect of the protons originating from the dissociated carboxyl groups. A schematic diagram of such transenantiomerization of profens via keto–enol tautomerism is shown in Fig. 10. The greater amplitude of oscillation at 6°C than at 22°C, apparent from both the $R_F = f(t)$ and the $[\alpha]_D = f(t)$ plots, seems to originate from the fact that reducing the temperature usually stabilizes short-living tautomers. This seems to be indirect confirmation of the correctness of our assumption of the molecular-level mechanism of the observed transenantiomerization. This assumption does not, however, explain the oscillatory nature of the phenomenon discussed.
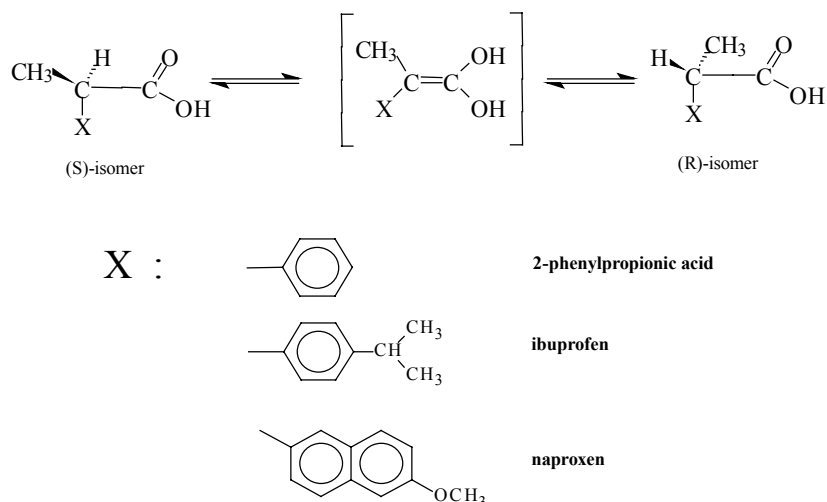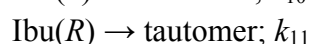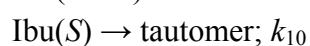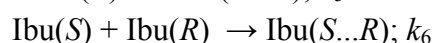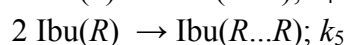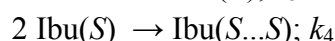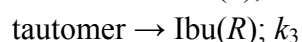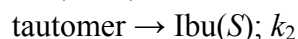
Fig. 10

Schematic representation of transenantiomerization of profens by keto–enol tautomerism

Let us reflect for a while on this behaviour. There is no doubt that the Belousov–Zhabotinskii (B–Z) reaction is the most famous example of such behaviour, probably because it was noticed and described first. Contrary to widespread conviction however, oscillatory reactions are not a rare exception, even though few have yet been reported and still fewer thoroughly elucidated [10]. In fact, oscillations can happen in reactions with non-linear kinetics and are not possible in those with linear kinetics only (i.e. monomolecular reactions with linear rate equations of the type $da/dt = ka$). Needless to say, the free energy even of oscillatory systems decreases continuously during the course of the reaction, i.e. ΔG does not oscillate, and there is evidently no deviation of these systems from the general rules of thermodynamics. Autocatalysis is one factor that can play central role in driving the oscillations and it is often combined in a larger mechanism with a set of other elementary steps. It seems very probable that transenantiomerization via acidic autocatalysis and the keto–enol tautomerism (Fig. 10) is responsible for these steric/optical oscillations, observed by means of polarimetry and thin-layer chromatography for all three profens.

A larger mechanism involved in transenantiomerization of profens can be described by means of a set of eleven elementary steps. In the equa-

tions below – for greater clarity – we restrict ourselves to descriptors (e.g. Ibu(S), Ibu(R)) which refer to ibuprofen only:

Ibu($S...S$) $\rightarrow$ tautomer + Ibu(S); k_1
tautomer $\rightarrow$ Ibu(S); k_2
tautomer $\rightarrow$ Ibu(R); k_3
2 Ibu(S) $\rightarrow$ Ibu($S...S$); k_4
2 Ibu(R) $\rightarrow$ Ibu($R...R$); k_5
Ibu(S) + Ibu(R) $\rightarrow$ Ibu($S...R$); k_6
Ibu($R...R$) $\rightarrow$ tautomer + Ibu(R); k_7
Ibu($S...R$) $\rightarrow$ tautomer + Ibu(S); k_8
Ibu($S...R$) $\rightarrow$ tautomer + Ibu(R); k_9
Ibu(S) $\rightarrow$ tautomer; k_{10}
Ibu(R) $\rightarrow$ tautomer; k_{11}

where Ibu(S) and Ibu(R) are, respectively, S-(+)- and R-(−)-ibuprofen; Ibu(S…S), Ibu(R…R), and Ibu(S…R) are, respectively, cyclic dimers involving two S species, two R species, and one S and one R species; and k_i is the rate constant of the ith elementary reaction (where $i = 1, 2, …, 11$).

By solution of the rate equations for this set of eleven elementary steps the oscillatory transenantiomerization of the three profens can most probably be modelled, although it is not absolutely certain whether we have managed to take into account all the elementary steps involved. Then the network of the elementary reactions participating in the process of structural transformation can be changed and, finally, kinetic study of the oscillatory instability of the profen enantiomers selected will possibly provide an ultimate elucidation of this striking and important phenomenon. This will be the next step in our study of this oscillatory instability.

CONCLUSIONS

TLC is a sufficiently well performing separation technique to enable satisfactory separation of enantiomer pairs (which is the question of a sufficient number of theoretical plates (N) in thin-layer chromatographic systems). There are, however, enantiomer pairs (for example the profens), which cannot be separated even with aid of the best performing separation tools. TLC can then prove a versatile multipurpose experimental tool (in qualitative terms comparable, e.g., with polarimetry), applicable as an al-

ternative solution for diverse experimental tasks from the field of physical chemistry.

It seems likely that the oscillatory changes of the enantiomeric configuration of profens are very likely to occur not only under laboratory conditions, but also at the pharmacokinetic and the drug-interaction stage in the human body. In view of our recent experimental results, the scientific reports claiming clearly predominant curing potential of the profen S enantiomers (e.g. Refs. [11–15]) become less convincing and for this reason certainly need to be carefully re-examined and re-considered.

ACKNOWLEDGEMENT

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