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Enantioseparation of Flurbiprofen and Ketoprofen in Patches and in Urine Excretions by Achiral Gas Chromatography

Man-Jeong Paik, Duc-Toan Nguyen, and Kyoung-Rae Kim
College of Pharmacy, Sungkyunkwan University, Suwon 440-746, Korea

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The enantiomeric composition tests on flurbiprofen and ketoprofen present in patch products and in urine excretions following patch applications were performed as diastereomeric (*R*)-(+)-1-phenylethylamides by achiral gas chromatography and by gas chromatography-mass spectrometry in selected ion monitoring mode. The method for determination of (*R*)- and (*S*)-enantiomers in the range from 0.1 to 5.0 μg was linear ($r \geq 0.9996$) with acceptable precision (% RSD ≤ 5.2) and accuracy (% RE = 0.6 ~ -2.4). The enantiomeric compositions of flurbiprofen in one patch product and of ketoprofen in five different products were identified to be racemic with relatively good precision (≤ 6.4%). The urinary excretion level of (*R*)-flurbiprofen was two times higher than its antipode, while the comparable excretion levels of (*R*)- and (*S*)-enantiomers for ketoprofen were observed.

Key words: Enantioseparation, Flurbiprofen, Ketoprofen, Patch, Gas chromatography, Gas chromatography-mass spectrometry in selected ion monitoring

INTRODUCTION

The antiinflammatory activities of flurbiprofen, 2-(2-fluoro-4-biphenyl)-propionic acid, and ketoprofen, 2-(4-isobutylphenyl) propionic acid, are ascribed to (*S*)-(+)-enantiomer (Lombardino, 1985; Jamali and Wainer, 1993). However, they are mostly marketed as racemic mixtures, though some of their antipodes, (*R*)-(-)-enantiomers exhibit different toxicological and pharmacological properties (Tracy and Hall, 1992; Jamali and Wainer, 1993; Gareil, 1996; Glowka and Karazniewicz, 2004; Levoin *et al.*, 2004). Hence, the production of active (*S*)-flurbiprofen and (*S*)-ketoprofen in enantiomerically pure form, their optical purity control and stereoselective pharmacokinetic study have become important tasks (Davis, 1997; Bhunshan *et al.*, 1998). Unlike ibuprofen and fenoprofen, (*R*)-ketoprofen undergoes a small degree of inversion to (*S*)-enantiomer (Grubb *et al.*, 1996; Blanco *et al.*, 1998a; Levoin *et al.*, 2004) and (*R*)-flurbiprofen undergoes no observable transformation (Gareil, 1996). Therefore, they are mostly used in patch forms for the treatment of rheumatoid arthritis and its related conditions by applying directly to the diseased sites or on the inflamed sites for reducing side effects of other organs (Rhee *et al.*, 1999; Oh *et al.*, 2001).

Most of the high-resolution methods developed for enantioseparation of ketoprofen and flurbiprofen employ capillary electrophoresis (Blanco *et al.*, 1998a, 1998b; Abushoffa *et al.*, 2002; Blanco *et al.*, 2003; La *et al.*, 2004) and high performance liquid chromatography (Lovlin *et al.*, 1996; Boisvert *et al.*, 1997; Ameyibor *et al.*, 1998; Teng *et al.*, 2003). However, gas chromatography (GC) combined with mass spectrometry (MS) employing achiral capillary columns with incomparably higher resolving power and long-term durability was found to offer rapid and robust enantioseparation of profens as diastereomeric derivative with positive peak identification suitable for pharmacokinetic studies in our recent reports (Paik *et al.*, 2004; Paik and Kim, 2004). Eight profens were rapidly (within 3 min) converted to diastereomeric (*R*)-(+)-1-phenylethylamide derivatives with no evidence of racemization. Moreover, the achiral GC system provided simultaneous enantioseparation of eight profens including flurbiprofen and ketoprofen in a single run with high enantioresolution within 30 min. When applied to commercial (*S*)-ibuprofen tablets, the optical purities (98.7~99.1%) were determined with good precision (% RSD ≤ 4.0). However, this achiral GC method has rarely been employed

Correspondence to: Kyoung-Rae Kim, College of Pharmacy, Sungkyunkwan University, Suwon 440-746, South Korea
Tel: 82-31-290-7703, Fax: 82-31-290-7723
E-mail: krkim@skku.edu

in enantiomeric composition tests for flurbiprofen and ketoprofen in commercial patches and in chiral discrimination of their urinary excretions following topical applications.

The present work was undertaken to extend the previous achiral GC method (Paik and Kim, 2004) to the enantiomeric composition tests of flurbiprofen in one brand and ketoprofen in five different brands of patches after converting into diastereomeric (*R*)-(+)-1-phenylethylamide derivatives. And the metabolic conversions of (*R*)-enantiomers occurred after topical patch applications were estimated by measuring enantiomeric ratios in urine excretions by GC-MS with selected ion monitoring (SIM) mode. Prior to enantioseparation, the chemical identity and purity of each profen extracted from the patch matrices were confirmed as *tert*-butyldimethylsilyl (TBDMS) derivatives (Kim *et al.*, 1993).

MATERIALS AND METHODS

Chemicals

Racemic flurbiprofen and ketoprofen, (*S*)-flurbiprofen and (*S*)-ketoprofen, 3-phenylpropionic acid, (*R*)-(+)-1-phenylethylamine (-1-PEA), triethylamine (TEA) and ethyl chloroformate (ECF) were obtained from Sigma-Aldrich (St. Louis, MO, USA). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA). Diethyl ether, acetonitrile, ethyl acetate, toluene, dichloromethane of spectroanalyzed grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were of analytical-reagent grade and used as received.

Preparation of standards, reagent and aqueous calibration solutions

Each stock solution of (*S*)-flurbiprofen and (*S*)-ketoprofen, racemic flurbiprofen and ketoprofen, and 3-phenylpropionic acid used as internal standard (IS) was made up at 10 mg/mL in acetonitrile as their free acid forms. The working solutions were then prepared by diluting each stock solution to 50 μg/mL with acetonitrile. (*R*)-(+)-1-PEA solution was prepared at 0.5 M in methanol. TEA and ECF solutions were prepared in acetonitrile at 50.0 and 60.0 mM, respectively. Five calibration samples for the measurement of flurbiprofen and ketoprofen enantiomers were prepared to contain each enantiomer in the range of 0.1 to 5.0 μg with racemic flurbiprofen and ketoprofen working solutions. All standard solutions prepared were stored at 4 °C.

Gas chromatography and gas chromatography-mass spectrometry

The GC analyses were performed with an Agilent 6890 gas chromatograph equipped with a split/splitless inlet system, flame ionization detector (FID) and GC Chemstation (Agilent Technologies, Atlanta, GA, USA). The injector was installed with a DB-17 MS (OV-17 bonded) fused-silica capillary column (15 m × 0.25 mm I.D., 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA). The injector and detector temperatures were 260 and 290 °C, respectively. Samples (*ca* 1.0 μL) were injected in the splitless mode with purge delay time of 0.7 min. The inlet pressure of helium as carrier gas was set to 45 kPa. The oven temperature was initially at 150 °C (1 min) and programmed at 30 °C/min to 260 and finally to 290 °C (15 min) at 10 °C/min.

GC-MS analyses were performed with an Agilent 6890 gas chromatograph interfaced to an Agilent 5973 mass-selective detector (70 eV, electron impact mode) and installed with an Ultra-2 (SE-54 bonded phase; 25 m× 0.20 mm I.D., 0.11 μm film thickness) cross-linked capillary column (Agilent Technologies, Atlanta, GA, USA). The temperatures of injector, interface and ion source were 260, 300 and 230 °C, respectively. Helium was used as carrier gas at a flow rate of 0.5 mL/min with constant flow mode. Samples were introduced in the split-injection mode (10:1) and the oven temperature was maintained at 150 °C (2 min) and programmed to 250 °C at 20 °C/min and finally to 300 °C (10 min) at a rate of 5 °C/min. The mass range scanned was 50-650 u at a rate of 0.99 scan/s. For the identification of urinary profen enantiomers in SIM mode, the following three ions for each analyte were selected: ions at m/z 238, 253 and 254 for IS, ions at m/z 199, 200 and 347 for flurbiprofen, ions at m/z 209, 210 and 357 for ketoprofen. The molecular ions at m/z 254, 347 and 357 were used for the quantitation of IS, flurbiprofen and ketoprofen, respectively.

Diastereomeric (*R*)-(+)-1-phenylethylamide formation

Flurbiprofen and ketoprofen were subjected to (*R*)-(+)-1-phenylethylamidation reaction as described elsewhere (Paik and Kim, 2004). Briefly, each sample containing flurbiprofen or ketoprofen was added with IS at constant amount (500 ng) and evaporated to dryness (under gentle nitrogen stream). The residue was dissolved in dichloromethane (200 μL) and then sonicated (1 min) after addition of 0.06 μmol of TEA (50 mM, 1.2 μL) and 0.24 μmol of ECF (60 mM, 4 μL). Subsequently, 0.5 M (*R*)-(+)-1-PEA (2 μL) was added and sonicated (2 min). After acidification (pH ≤2) with 0.1 M hydrochloric acid (200 μL), the amide derivatives were extracted with diethyl ether (600 μL) and ethyl acetate (600 μL) in sequence. The combined extracts were evaporated to dryness (under gentle nitrogen stream) and the residue was reconstituted in a mixture (20 μL) of toluene and ethyl acetate (1:1) for GC and GC-MS analysis.

Calculations of corrected peak area ratios for quantitation

The quantitative calculations of flurbiprofen and ketoprofen enantiomers were based on corrected peak area ratios relative to that of IS. The optical purity of (*R*)-(+)-1-PEA was measured to be 99.9% with high precision (better than ± 0.1% RSD) employing *N*-ethoxycarbonyl-L-alanine as chiral resolving agent in the previous study (Paik *et al.*, 2004). Hence, the peaks corresponding to (*R*)-flurbiprofen and (*R*)-ketoprofen contained (*S*)-flurbiprofen and (*S*)-ketoprofen at 0.1%, respectively. In the same manner, the (*S*)-flurbiprofen and (*S*)-ketoprofen peaks contained (*R*)-flurbiprofen and (*R*)-ketoprofen at 0.1%, respectively. The peak area ratios of (*R*)-enantiomers and (*S*)-enantiomers were thus corrected according to the following equations in all calculations (Paik and Kim, 2004) :

Corrected peak area ratio for (*R*)-enantiomer
$= (0.999\ X + 0.001\ Y) / Z$
Corrected peak area ratio for (*S*)-enantiomer
$= (0.001\ X + 0.999\ Y) / Z$

Here X and Y were the measured peak areas of (*R*)-enantiomer and (*S*)-enantiomer, respectively, while Z was the peak area of IS.

Method validation for enantiomeric assay of flurbiprofen and ketoprofen

Method validations for the measurements of flurbiprofen and ketoprofen enantiomers were conducted with five calibration samples (0.1, 0.5, 1.0, 2.5, and 5.0 μg) made of respective racemic standards at constant amount (0.5 μg) of IS. Linearity was tested by least-squares regression analysis on the corrected peak area ratios against increasing amount ratios of each enantiomer. The precision expressed as percentage of relative standard deviation (% RSD) and accuracy as percentage of relative error (% RE) of the method were determined at three different amounts (0.5, 1.0, and 5.0 μg) for each enantiomer present in racemic standards in triplicate.

Extraction of flurbiprofen and ketoprofen from patches in free acid forms

One brand of flurbiprofen patch containing 20 mg of flurbiprofen and five different brands of ketoprofen patch, each containing 30 mg of ketoprofen were locally purchased. An aliquot (ca 0.67 g) from finely cut patches (≤ 2~3 mm length) of each profen was vortex-mixed with alkaline water (2.5 mL, pH ≥12) for 10 min. The aqueous extract containing profen in ionized form was washed with diethyl ether (3 mL × 5), followed by acidification (pH ≤2) and saturation with sodium chloride. The profen in free acid form was extracted with diethyl ether (3 mL × 3) and evaporated to dryness (under nitrogen gentle stream). Each sample stock solution in free acid form was made up at 5 mg/mL in acetonitrile and the working sample solution was prepared by diluting the stock solution to 100 μg/mL with acetonitrile.

Analysis of flurbiprofen and ketoprofen in patch extracts as racemic *tert*-butyldimethylsilyl derivatives

An aliquot (5.0 μg) of each patch extract was evaporated to dryness (under gentle stream of nitrogen) and added with TEA (5 μL) and toluene (10 μL). The mixture was then reacted with MTBSTFA (10 μL) at 60 °C for 1 h to form TBDMS derivatives with subsequent analysis by GC and GC-MS.

Enantioseparation of flurbiprofen and ketoprofen in patch extracts as (*R*)-(+)-1-phenylethylamide derivatives

An aliquot (5.0 μg) of each patch extract was added with IS (0.5 μg) and evaporated to dryness. The residue was then subjected to diastereomeric (*R*)-(+)-1-phenylethylamide formation with subsequent GC and GC-MS analysis as described in the preceding sections.

Sample preparation for measurements of flurbiprofen and ketoprofen enantiomers in urine samples

Urine samples were individually collected at 9 h after applying flurbiprofen patch to one healthy male volunteer and ketoprofen patch (product No. 2) to another male volunteer. An aliquot (2 mL) of each urine sample after addition IS (0.5 μg) was adjusted to pH ≥12 (with 5.0 M sodium hydroxide) and washed with diethyl ether (3 mL× 2). The aqueous phase was then acidified (pH ≤2.0) and saturated with sodium chloride, followed by extraction with diethyl ether (3 mL × 2). The combined extracts were evaporated to dryness and the residue was subjected to diastereomeric (*R*)-(+)-1-phenylethylamide formation as described in the preceding sections for the analysis by GC-MS in SIM mode

RESULTS AND DISCUSSION

Method validation for measurement of flurbiprofen and ketoprofen enantiomers

From a number of preliminary experiments, each optimal amount of TEA, ECF and (*R*)-(+)-1-PEA used for ibuprofen as reported elsewhere (Paik and Kim, 2004) was found to be optimal for the (*R*)-(+)-1-phenylethylamidation of flurbiprofen and ketoprofen. Under the present GC condition, complete enantioseparations of racemic flurbiprofen and ketoprofen standards were achieved and

the impurity peaks originated from solvents and reagents did not interfere with measurement of each enantiomer.

When the detector response (expressed as peak area ratios) of each resolved enantiomer at microgram levels employing calibration samples made of racemic flurbiprofen and ketoprofen standards were plotted against increasing amounts of 0.1~5.0 μg (expressed as weight ratios) for each enantiomer, good linear relationship ($r \geq 0.9996$) was obtained (Table I). The precisions (% RSD) and accuracy (% RE) of the method measured at three different amounts (0.5, 1.0, and 5.0 μg) were varied from 1.4 to 5.2 and from 0.6 to -2.4, respectively. These results indicated that the level of each enantiomer present in racemates could be measured with good precision and accuracy.

Chemical identification and enantiomeric compositions of flurbiprofen and ketoprofen in patch extracts

The chemical identifications of flurbiprofen and ketoprofen extracted from patches were carried out as their racemic TBDMS derivatives. A single peak corresponding to each racemate was obtained with minor reagent impurities for flurbiprofen (Fig. 1-A-1) and ketoprofen (Fig. 1-B-1). This indicated that the present simple two-step extraction method was effective in recovery of each profen with high purity from patch matrices.

Upon their chiral analysis as diastereomeric (*R*)-(+)-1-phenylethylamide derivatives, complete enantioseparation of flurbiprofen (Fig. 1-A-2) and ketoprofen (Fig. 1-B-2) were achieved. The amounts of (*R*)- and (*S*)-enantiomers in an aliquot (5 μg) of each patch extract were measured with satisfactory precision (≤ 6.4%) (Table II). All of the patch products were virtually in racemic composition (50:50) except for the flurbiprofen patch (No. 1) which had a little less amount of active (*S*)-enantiomer.

Enantioseparation of flurbiprofen and ketoprofen in urine excretions following patch application

Because of the co-extracted multiple endogenous organic acids at much higher concentrations, screening for acidic profens at sub-microgram levels in urine specimens required the use of GC-MS in SIM mode as reported previously (Kim and Yoon, 1996). The present GC-MS-SIM analysis provided a very selective monitoring condition for each enantiomer of flurbiprofen (Fig. 2-A) and ketoprofen (Fig. 2-B) excreted at 9 h after applying each patch. The composition ratio of (*R*)-enantiomer to (*S*)-enantiomer for flurbiprofen for the triplicate runs was calculated to be at 65.6 (±0.9) : 34.4 (±0.9) based on their corrected peak area ratios. The two times higher excretion level of (*R*)-enantiomer might be due to no appreciable occurrence of metabolic inversion as reported in

Table I. Linearity, precision and accuracy for the measurement of flurbiprofen and ketoprofen enantiomers as their (*R*)-(+)-1-phenylethylamides

Profen	Calibration range (μg)[a]	Regression line		r^d	Amount added (μg)	Precision (% RSD)[e]	Accuracy (% RE)[f]
		m^b	b^c				
(*R*)-Flurbiprofen	0.1-5.0	0.67 ± 0.01	0.03 ± 0.05	0.9997	0.5	2.7	-2.2
					1.0	2.5	-0.9
					5.0	2.0	-0.8
(*S*)-Flurbiprofen	0.1-5.0	0.67 ± 0.01	0.02 ± 0.05	0.9996	0.5	4.2	0.6
					1.0	2.9	-1.2
					5.0	1.4	-1.0
(*R*)-Ketoprofen	0.1-5.0	0.510 ± 0.005	0.02 ± 0.03	0.9998	0.5	3.4	-1.9
					1.0	3.7	0.2
					5.0	3.8	-0.6
(*S*)-Ketoprofen	0.1-5.0	0.495 ± 0.007	0.02 ± 0.04	0.9997	0.5	5.2	-2.4
					1.0	4.8	-0.6
					5.0	3.6	-0.8

[a] Calibration range corresponding to each enantiomer in racemic standards
[b] Slope; (mean ± standard deviation)
[c] Intercept; (mean ± standard deviation)
[d] Correlation coefficient
[e] Relative standard deviation for n = 3
[f] Relative error; {(measured mean value - nominal value) / nominal value} × 100
All quantitative calculations were based on peak area ratios relative to that of IS (3-phenylpropionic acid, 0.5 μg) measured on DB-17 MS column in triplicate

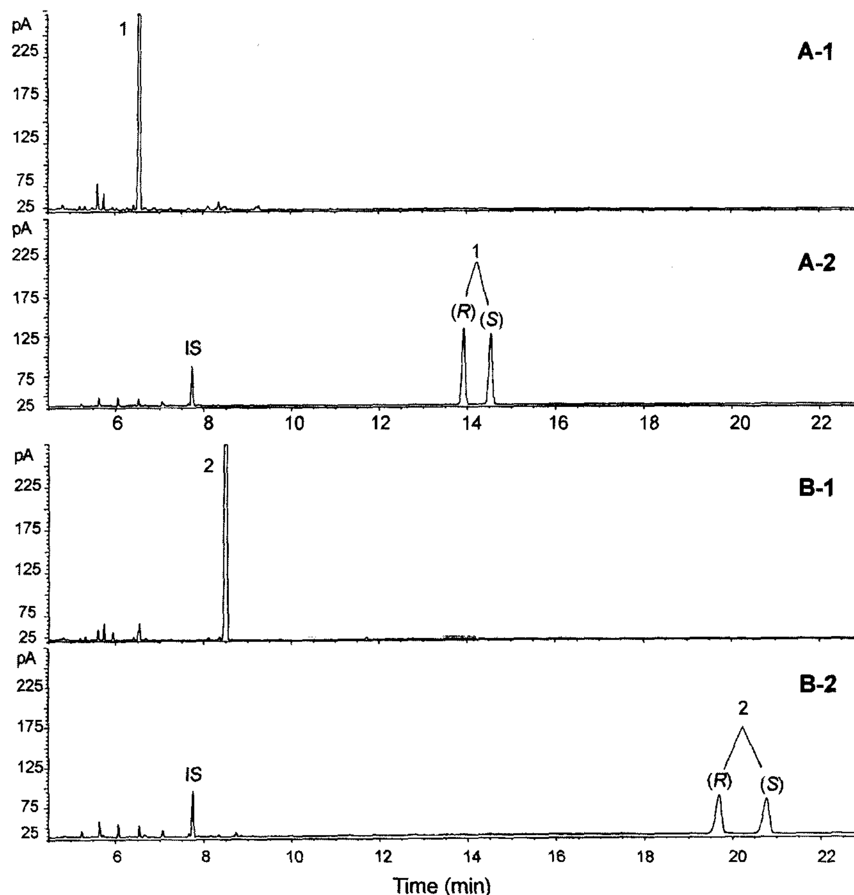

Fig. 1. GC chromatograms of racemic *tert*-butyldimethylsilyl derivatives of flurbiprofen (A-1) and ketoprofen (B-1) and of diastereomeric (*R*)-(+)-1-phenylethylamide derivatives of flurbiprofen (A-2) and ketoprofen (B-2) in patch extracts separated on DB-17 MS column (OV-17 bonded phase; 15 m × 0.25 mm I.D., 0.25 μm film thickness). Peaks: 1 = flurbiprofen; 2 = ketoprofen; IS = 3-phenylpropionic acid. GC conditions are in the text.

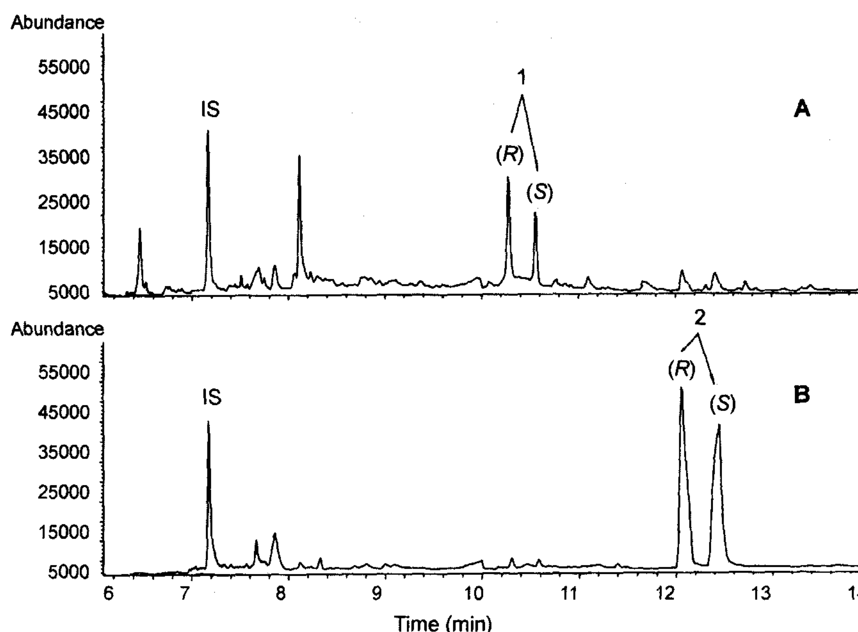

Fig. 2. Enantiomeric profiles of flurbiprofen (A) and ketoprofen (B) as diastereomeric (*R*)-(+)-1-phenylethylamides in urine excretions after patch application in GC-MS-SIM mode separated on Ultra-2 column (SE-54 bonded phase; 25 m × 0.20 mm I.D., 0.11 μm film thickness). Peaks: 1 = flurbiprofen; 2 = ketoprofen; IS = 3-phenylpropionic acid. GC-MS-SIM conditions are in the text.

Table II. Enantiomeric composition test on one flurbiprofen- and five different ketoprofen-patch products as their (*R*)-(+)-1-phenylethylamides

Product No.	Enantiomer	Amount (μg)[a]	Composition (%)
1	(*R*)-Flurbiprofen	2.55 ± 0.10 (3.9)	50.6 ± 0.2
	(*S*)-Flurbiprofen	2.48 ± 0.11 (4.4)	49.4 ± 0.2
2	(*R*)-Ketoprofen	2.43 ± 0.12 (4.9)	49.7 ± < 0.1
	(*S*)-Ketoprofen	2.46 ± 0.12 (4.9)	50.3 ± < 0.1
3	(*R*)-Ketoprofen	2.35 ± 0.14 (6.0)	49.8 ± 0.1
	(*S*)-Ketoprofen	2.37 ± 0.14 (5.9)	50.2 ± 0.1
4	(*R*)-Ketoprofen	2.48 ± 0.15 6.0)	49.9 ± 0.3
	(*S*)-Ketoprofen	2.50 ± 0.16 (6.4)	50.1 ± 0.3
5	(*R*)-Ketoprofen	2.57 ± 0.10 (3.9)	50.0 ± < 0.1
	(*S*)-Ketoprofen	2.56 ± 0.09 (3.5)	50.0 ± < 0.1
6	(*R*)-Ketoprofen	2.50 ± 0.08 (3.2)	49.9 ± 0.1
	(*S*)-Ketoprofen	2.51 ± 0.09 (3.6)	50.1 ± 0.1

[a] Amount (mean ± standard deviation for n = 3) calculated using regression equations in Table I

the literature (Gareil, 1996). In contrast, the comparable excretion levels of (*R*)- and (*S*)-ketoprofens with the estimated ratio of 53.5 ± (0.9) : 46.5 (± 0.9) might support the previous reports that a small degree of inversion undergoes (Grubb *et al.*, 1996; Blanco *et al.*, 1998a; Levoin *et al.*, 2004). The present results must be further confirmed by the stereospecific pharmacokinetic study on flurbiprofen and ketoprofen absorbed through dermal skin as racemates and as optically pure enantiomers.

CONCLUSIONS

The present method provided rapid determination of (*R*)-(-)- and (*S*)-(+)-enantiomers of flurbiprofen and ketoprofen as (*R*)-(+)-1-phenylethylamides in patch products and in urine excretions within 22 min by achiral GC, and within 13 min by GC-MS-SIM, respectively. The enantiomeric compositions of flurbiprofen in one patch product and of ketoprofen in five different products were measured to be racemic with acceptable precision (≤ 6.4%), indicating that the present method is suitable for quality assurance tests of other chiral profens and similar acidic drugs. The excretion levels of (*R*)-enantiomers of flurbiprofen and ketoprofen in urine following patch applications were measured to be higher compared to the respective antipodes. A further study is under way for the stereospecific pharmacokinetic investigations on the flurbiprofen and ketoprofen absorbed through dermal skin from their patches.

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