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Enantiospecific gas chromatographic–mass spectrometric procedure for the determination of ketoprofen and ibuprofen in synovial fluid and plasma: application to protein binding studies

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ABSTRACT

A method for the enantiospecific quantitation of two commonly prescribed non-steroidal anti-inflammatory drugs (ketoprofen and ibuprofen) is described. The method involves formation of a mixed anhydride of the drug with ethylchloroformate and subsequent conversion to an amide by reaction with optically active amphetamine. The subsequently formed diastereomers are separated by gas chromatography–mass spectrometry using selected-ion monitoring. The assay is capable of quantifying ketoprofen (2 ng/ml) and ibuprofen (3 ng/ml) enantiomers from a 200-μl sample of synovial fluid or plasma and is particularly suitable for protein binding studies.

INTRODUCTION

Interest in the pharmacokinetics of racemic non-steroidal anti-inflammatory drugs (NSAIDs) [1], such as ketoprofen and ibuprofen, and their relevance to clinical practice [2] has grown markedly over the last decade. From a pharmacological viewpoint, the prostaglandin synthetase inhibiting effect of these NSAIDs is attributable to the *S*(+)-enantiomers [3]. These compounds may undergo variable metabolic inversion from the *R*(−)-enantiomer to the corresponding *S*(+)-enantiomer by an unproven mechanism [1]. It has been claimed that the pharmacokinetic behaviour of the two enantiomers in humans may be different [1], so the stereoselective investigation of the pharmacokinetic parameters of these agents would appear to be of scientific and medical relevance. Furthermore, attention has begun to focus on the free or unbound NSAID, as this is the species which diffuses

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across membranes and binds to specific receptors. As large inter-individual differences in this free fraction have been reported [4] one might expect the clinical effects of such drugs to be more closely related to their unbound rather than total concentrations.

Investigation of these drugs at or near the presumed site of action (the joint) are often hampered by the volume of synovial fluid able to be collected from patients. As these NSAIDs are so extensively bound to plasma and synovial fluid proteins (≥99%), the need to measure unbound enantiomer drug levels of the order of 5 ng/ml using 500 μl or less of synovial fluid is not uncommon.

Whilst there have been numerous methods developed for the quantification of ketoprofen and ibuprofen in biological fluids [5–11], there have been relatively few attempts at resolving their respective enantiomers (Table I). These enantiomeric assays often involved lengthy and complex derivatization procedures associated with lengthy incubation periods. This paper reports a rapid derivatization with dexamphetamine using ethylchloroformate as the coupling reagent, followed by gas chromatography–mass spectrometry (GC–MS) using selected-ion monitoring (SIM) to give the sensitivity required for ketoprofen and ibuprofen enantiomeric protein binding studies in the clinical environment.

TABLE I

PREVIOUS ENANTIOMERIC ASSAYS

Author	Method	MQC[a] (ng/ml)	Sample volumes[b] (μl)
Ketoprofen			
Sallustio *et al.* [19]	HPLC	200	200
Foster and Jamali [16]	HPLC	50	500
Björkman [15]	HPLC	250	500
Ibuprofen			
Geisslinger and Dietzel [20]	HPLC	100	500
Singh *et al.* [17]	GC	75	1000
Avgerinos and Hutt[21]	HPLC	500	500
Lee *et al.* [14]	HPLC	500	1000
Van Giessen and Kaiser [22]	GC	500	1000

[a] MQC = minimum quantifiable concentration.

[b] Sample volume = volume plasma/synovial fluid required for assay.

EXPERIMENTAL

Reagents and chemicals

Samples of *S*(+)-, *R*(−)- and *R,S*-ibuprofen were the generous gift of Boots (Nottingham, UK). Ethylchloroformate (May and Baker, Dagenham, UK) was LR grade. All solvents were high-performance liquid chromatography (HPLC) grade (Waters Assoc., Sydney, Australia) except *n*-hexane, dichloromethane and toluene (all nanograde, Mallinckrodt, KY, USA), ^2H_2O (Aldrich, Milwaukee, WI, USA) and diethyl ether (LR grade, May and Baker). Stock solutions of *R,S*-ketoprofen (Sigma, St. Louis, MO, USA) and *R,S*-ibuprofen, 1 μg/ml in methanol, were each prepared monthly and stored at 4°C.

Dexamphetamine solution was prepared monthly by basification of 10 ml of dexamphetamine sulphate (Sigma) aqueous solution (equivalent to 750 μg free base per ml) to pH 11 by the addition of 100 μl of 2 *M* sodium hydroxide and subsequent extraction with 20 ml of diethyl ether. The organic phase was evaporated to dryness under a gentle stream of nitrogen gas and the residue reconstituted in 10 ml of methanol and stored at room temperature. Optical purity of dexamphetamine sulphate (≥99.3%) was reconfirmed at six monthly intervals by derivatization with pure (100.0%) optically active *S*(+)-naproxen (Sigma).

The extraction solvent mixture *n*-hexane–diethyl ether–isopropyl alcohol (80:20:0.1, v/v) was freshly prepared at weekly intervals.

Internal standards

Naproxen, *o*- and *p*-toluic acids (Sigma) were used during developmental stages of this assay. Stable isotope dilution methods were used thereafter. Deuterated *R,S*-ibuprofen was prepared by the method of Dawson *et al.* [12]. Deuterated *R,S*-ketoprofen was prepared from its non-deuterated counterpart via an electrophilic substitu-

tion reaction with deuterated sulphuric acid, using a modified method of a previous deuteration to benzene by Ingold *et al.* [13]. This involved careful dilution of 2.7 ml of $^2H_2SO_4$ (Novachem, Melbourne, Australia) with 900 μl of ^2H_2O and subsequent transfer of the diluted acid to a 5-ml glass ampoule (ACI, Sydney, Australia) containing 5 mg of *R,S*-ketoprofen. The ampoule was sealed under nitrogen, then heated at 115°C for 12 h. After cooling, the ampoule was opened, the contents transferred to a tapered glass tube (using 5 ml of extraction solvent), then washed with 10 ml of purified water. The organic phase was transferred to another tapered glass tube and evaporated to dryness. The residue was weighed, reconstituted in methanol (to a final concentration of 500 ng/ml), then stored at 4°C.

Aliquots of the deuterated materials (equivalent to 1 μg solid) were derivatized and analysed using the procedure described below. The deuterium content of the ketoprofen sample was d_0 (<0.1%), d_1 (0.4%), d_2 (3.0%), d_3 (9.7%), d_4 (21.5%), d_5 (25.4%), d_6 (21.8%), d_7 (12.1%), d_8 (4.7%) and d_9 (1.4%). For the ibuprofen sample, the deuterium content was d_0 (0.2%), d_1 (0.7%), d_2 (4.0%), d_3 (17.3%), d_4 (62.2%), d_5 (12.4%) and d_6 (3.2%). For both samples, the mass spectra indicated that the non-selective deuterium labelling was predominantly on the aromatic rings. The yields of ketoprofen and ibuprofen deuterated materials were 17 and 30%, respectively.

Instrumentation

The GC–MS system comprised a Hewlett-Packard 5890 gas chromatograph coupled to an HP5970B mass-selective detector and an HP59970A data system (Hewlett-Packard, MD, USA). A 25 m × 0.32 mm I.D. fused-silica column with a cross-linked methyl silicone-bonded stationary phase of film thickness 0.17 μm (HP-1, Hewlett-Packard) was used with helium carrier gas at a linear velocity of 55 cm/s at 50°C. For ketoprofen samples, the oven temperature was programmed from 60 to 230°C at 30°C/min and from 230 to 290°C at 10°C/min. For ibuprofen samples, the oven temperature was programmed from 60 to 200°C at 30°C/min, from 200 to 240°C at 8°C/min and from 240 to 290°C at 30°C/min. The split–splitless injector was operated in the splitless mode and fitted with a silanized wide-bore (4 mm) borosilicate liner that contained a small plug of glass wool in the centre. The injector temperature was 250°C and the transfer line temperature was 290°C. For both drugs, the instrument was tuned to *m/z* 219 for optimum sensitivity. Injection (1 μl) was by means of a Hewlett-Packard 7673A autosampler. The column was protected by a 20-cm length of a similar type of column (Econo-Cap SE-30: Alltech Assoc., IL, USA) which was fitted as a disposable pre-column.

Structures of the diastereomeric amides and deuterated internal standards were confirmed by electron-impact MS. Full-scan reference spectra of the amphetamine derivatives of both NSAID enantiomers were used to select appropriate ions for SIM during analytical runs.

Biological samples

A 200-μl aliquot of synovial fluid or plasma was transferred to a disposable glass centrifuge tube (Kimble, IL, USA), 100 μl of internal standard, 50 μl of 5 *M* HCl and 3 ml of extraction solvent were then added and the tube vortex-mixed for 1 min. The tube was then centrifuged (Jouan CT1000–Jouan SA-BP, St. Nazaire, France) for 5 min at 2500 *g* and 10°C, the supernatant evaporated to dryness under a gentle stream of nitrogen gas and the residue reconstituted with 100 μl of 50 mM triethylamine in acetonitrile. To this mixture (at 30-s intervals) were added 50 μl of 60 m*M* ethylchloroformate in acetonitrile and 50 μl of dexamphetamine solution. After 1 min, 0.5 ml of chilled water was added, followed by 3 ml of dichloromethane. The mixture was vortex-mixed for 30 s and centrifuged for 5 min at 2000 *g*. The organic layer was transferred to a tapered glass tube and evaporated to dryness with nitrogen. The residue was reconstituted in 20 μl of toluene, transferred to a small, tapered conical glass insert (Microsun Insert, Sun Brokers, Wilmington, USA) and placed within a 1.5-ml crimp-top glass autosampler vial (Sun Brokers). A 1-μl aliquot of this sample was injected into the GC–MS system.

The derivatization reaction time and reagent quantities were optimised using samples containing 40 ng of *S*-ibuprofen and 1 μg of *p*-toluic acid (prepared from their respective stock solutions). These were derivatized using varied reaction times (0.25–10 min) and varied volumes of dexamphetamine solution (20–300 μl).

Calibration curves

Seeded samples of ketoprofen (2 ng/ml to 50 μg/ml) and ibuprofen (3 ng/ml to 50μg/ml) were prepared by spiking "blank" synovial fluid and plasma with predetermined amounts of racemic stock solutions, followed by extraction and derivatization as above.

Quantitation was based on peak-area ratio [*m/z* 232 (ibuprofen) to *m/z* 236 (d_4-*S*-ibuprofen)] or peak-height ratio [*m/z* 209 (ketoprofen) to *m/z* 214 (d_5-*S*-ketoprofen)]. An unweighted least-squares regression was fitted to each individual calibration curve.

Recovery

The recovery of derivatized material per sample was assessed by comparison of the peak height (ketoprofen) and peak area (ibuprofen): deuterated internal standard ratios of five extracted samples of each drug (1 μg/ml) with those of five similarly prepared, non-extracted drug samples.

Clinical studies

These studies were conducted in human volunteers with effusions into the knee. The study was approved by the University Ethics Committee, and each subject gave informed, signed, witnessed consent.

A single 400-mg dose of *R,S*-ibuprofen (Brufen, Boots, Sydney, Australia) or 100 mg of *R,S*-ketoprofen (Orudis, May and Baker, Melbourne, Australia) was given to fasting patients with a glass of water. Blood samples (5 ml) were collected via a venous catheter inserted in a forearm vein, whilst 1 ml of synovial fluid samples were collected by aspiration of the knee joint at regular intervals over 24 h. Plasma was immediately separated by centrifugation in heparinised tubes. All samples were acidified and internally standardised within 8 h of collection, then derivatized and stored at 4°C until analysis.

Free (non-protein bound) drug samples

These were obtained by ultrafiltration of 500 μl of synovial fluid or 1 ml of plasma using the Amicon MPS-1 ultrafiltration system (Amicon, MA, USA). A 200-μl aliquot of ultrafiltrate from the device was acidified, internally standardised, derivatized and stored at 4°C until assayed. Samples were prepared in duplicate.

RESULTS AND DISCUSSION

Identification of enantiomers

Typical SIM chromatograms for both drugs are shown in Fig. 1. We initially used peak-area ratios for quantitation of both drugs, but later discovered slight tailing of ketoprofen peaks could vary unpredictably – apparently in sympathy with the number of samples per analytical run or with the state of cleanliness of the injection liner and pre-column. We therefore used peak-height ratios for ketoprofen quantitation.

Two peaks were resolved at 8.86 min (*S*) and 8.96 min (*R*) for ibuprofen diastereomers and at 11.78 min (*S*) and 11.90 min (*R*) for ketoprofen diastereomers. For ibuprofen, SIM of *m/z* 232 gave the cleanest trace, with freedom from interfering peaks, whilst *m/z* 209 was best for ketoprofen. In addition, *m/z* 280 (ketoprofen) and *m/z* 161 (ibuprofen) were also monitored, particularly to check for potential interfering peaks in weak samples (containing less than 50 ng/ml of either enantiomer).

Extraction solvent

A number of extraction solvents were considered. A study by Lee *et al.* [14] compared numerous extraction solvents from previous NSAID assays. They settled upon 0.1% isopropyl alcohol in *n*-hexane as the most suitable for their studies with ibuprofen. Using plasma and synovial fluids spiked with 100 ng/ml racemic ketoprofen or ibuprofen we found our optimum yield (≥90%) with minimal interfering peaks was obtained with

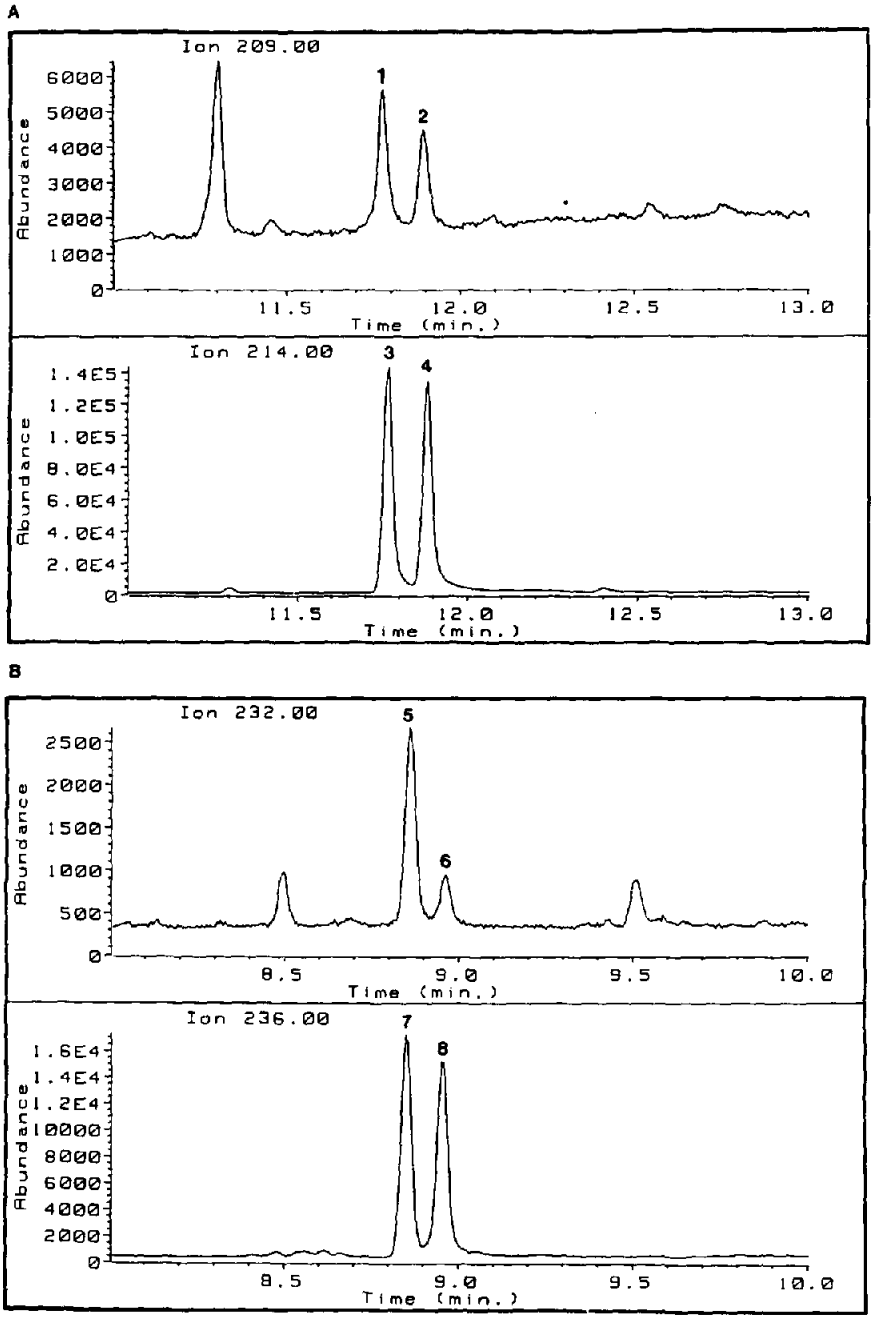

Fig. 1. (A) SIM chromatograms from a synovial fluid ultrafiltrate of an arthritic patient 12 h after a single oral dose of 100 mg of *R,S*-ketoprofen. Drug levels were: 13 ng/ml *S*-ketoprofen (peak 1) and 9 ng/ml *R*-ketoprofen (peak 2). The internal standard *S*-d_5-ketoprofen (peak 3) and *R*-d_5-ketoprofen (peak 4) are also shown. (B) SIM chromatograms from a plasma ultrafiltrate of an arthritic patient (on chronic ibuprofen drug therapy), 6 h after an oral dose of 400 mg of *R,S*-ibuprofen. Drug levels were: 54 ng/ml *S*-ibuprofen (peak 5) and 13 ng/ml *R*-ibuprofen (peak 6). The internal standard *S*-d_4-ibuprofen (peak 7) and *R*-d_4-ibuprofen (peak 8) are similarly shown.

the solvent mixture *n*-hexane–diethyl ether–isopropyl alcohol (80:20:0.1, v/v).

Optimisation of derivatization

Ethylchloroformate is a commonly used reagent for amide formation in peptide synthesis and when used in conjunction with *l*-leucinamide (for subsequent HPLC analysis) the coupling reaction has been reported to be complete in less than 3 min [15,16]. Consistent with these findings, we noted negligible variation in peak-area ratio after 60 s reaction time with the dexamphetamine derivatizing reagent.

Whilst a large excess of dexamphetamine theoretically remains after biological sample derivatization, a volume of 50 μl (equivalent to 100 μg free base) was settled upon (based on negligible yield variation with increasing volume of reagent used). This was twice the amount used by Singh *et al.* [17] in which dexamphetamine was coupled in a relatively slow (2 h) reaction using 1,1'-carbonyldiimidazole.

Internal standards

Whilst *o*- and *p*-toluic acids were satisfactory internal standards during the inital developmental stages of this assay, they proved less than ideal in biological samples, as clean chromatographic

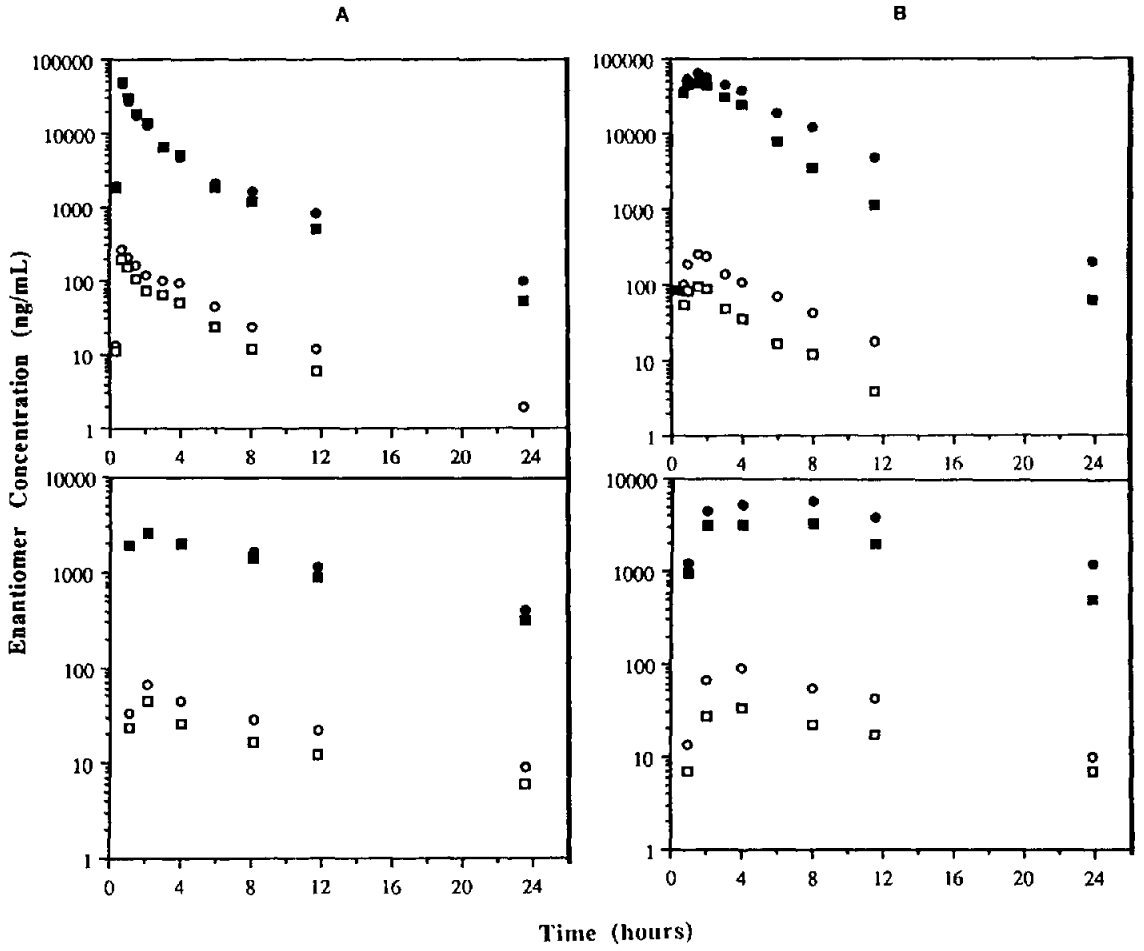

Fig. 2. Plasma and synovial fluid concentration–time profiles of total *S* (●), total *R* (■), free *S* (○) and free *R* (□) enantiomers in plasma (top windows) and synovial fluid (lower windows) of two arthritic patients following a single oral dose of 100 mg of *R,S*-ketoprofen (A) or 400 mg of *R,S*-ibuprofen (B).

traces could not be consistently obtained. In addition, we noted variations in the extraction efficiency of ibuprofen as compared to *o*- and *p*-toluic acids. To avoid these problems naproxen (being a larger molecule and more akin to ibuprofen than *o*- and *p*-toluic acids) was considered a potentially more suitable alternative internal standard for the ibuprofen assay. This was not to be the case for ketoprofen as repeated injections of the same drug sample over a three-day period led to variability in the 209 (ketoprofen) to 185 (naproxen) *m/z* ratio of $\geq 40\%$. When methyl stearate was incorporated as an independent internal standard, the consistency of the *m/z* 209/280 and 185/213 ratios (for *S*-ketoprofen and methyl stearate, respectively) removed any blame from the MS system. More likely, the problem lay with either the chromatography (increased irreversible adsorption of naproxen relative to ketoprofen) or possibly decomposition of the naproxen derivative itself. Either way, these variable on-column losses were unacceptable and best dealt with by use of deuterated internal standards. Reproducibility and stability problems seen with ketoprofen (and to a much lesser extent with ibuprofen) were then effectively dealt with.

Quantitation

The calibration curves for ibuprofen were linear over the entire concentration range studied (3 ng/ml to 50 μg/ml; $r \geq 0.99$). The detection limits for the ibuprofen enantiomers were less than 1 ng/ml whilst the minimum quantifiable concentration from a 200-μl sample of synovial fluid was 3 ng/ml.

The calibration curves for ketoprofen enantiomers (2 ng/ml to 50 μg/ml) were also linear ($r \geq 0.99$). The detection limit were less than 1 ng/ml whilst the minimum quantifiable concentration from a 200-μl sample of synovial fluid was 2 ng/ml.

This enhanced sensitivity over previous assays (Table I) allowed quantification of very low levels (<25 ng/ml) of unbound enantiomers of these drugs (Fig. 2) in synovial fluid and plasma.

Precision

The precision of the assays for ketoprofen and ibuprofen enantiomers at high and low concentrations were within acceptable limits for our intended clinical studies (Table II). In conjunction with the ultrafiltration procedure, the coefficient of variation for determination of ibuprofen enantiomers ($\leq 3.8\%$ *S*- and *R*-ibuprofen) over the entire concentration range studied compared favourably with the 10% variation of ibuprofen enantiomers obtained in the GC–MS assay procedure of Singh *et al.* [17].

TABLE II

PRECISION OF THE KETOPROFEN/IBUPROFEN ASSAYS

Drug	*n*	Theoretical concentration (ng/ml)	Experimental concentration (mean ± S.D.) (ng/ml)	C.V.[a] (%)
S(+)-ketoprofen	9	37.5	36 (2.5)	6.9
		5000	4596 (265.8)	5.8
R(−)-ketoprofen	9	37.5	32 (2.3)	7.2
		5000	4598 (279.3)	6.1
S(+)-ibuprofen	10	25	32 (1.2)	3.8
		750	772 (9.8)	1.3
R(−)-ibuprofen	10	25	26 (1.0)	3.8
		750	716 (9.8)	1.4

[a] C.V. = coefficient of variation.

TABLE III

SAMPLE STABILITY OVER 48 h

Derivative	Experimental concentration (ng/ml)	
	1 h	48 h
S(+)-Ketoprofen	94	93
	527	545
R(−)-Ketoprofen	95	93
	535	551
S(+)-Ibuprofen	45.2	40.5
	95.2	95.2
	247.6	216.7
R(−)-Ibuprofen	46.9	42.9
	93.9	95.9
	234.7	210.2

Similarly, for ketoprofen enantiomers, we obtained coefficients of variation of $\leqslant 6.9\%$ and $\leqslant 7.2\%$ for *S*- and *R*-ketoprofen, respectively.

Sample stability

Whilst all samples in this current work were generally analysed within 24 h of derivatization, the data clearly demonstrated that the influence of short-term sample storage on assay reproducibility was negligible (Table III). This is consistent with work by Testa [18] who demonstrated a general degree of increased stability of diastereomeric amide derivatives over that of esters for bioanalytical work.

CONCLUSIONS

The GC–MS assay procedure allowed quantitation of ketoprofen and ibuprofen enantiomers with a high degree of specificity at very low levels in synovial fluid and plasma. Fig. 2 illustrates typical plasma and synovial fluid concentration *versus* time curves for the enantiomers of ketoprofen and ibuprofen in two patients over a 24-h period. These results were achieved with the use of an open split interface in which up to 50% of the column effluent was not transferred to the detector; the use of a direct interface should enable improvements in signal strengths and hence even lower detection limits. We found our procedure was sufficiently sensitive for quantitation of samples typically encountered in protein binding studies. Perhaps equally important, the method allowed practical analysis of large numbers of patient samples (common to pharmacokinetic studies), owing to the relative speed of sample preparation and the ability to semi-automate the analytical work on a suitably equipped instrument.

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