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Determination of (*R*)- and (*S*)-ketoprofen in human plasma by liquid chromatography/tandem mass spectrometry following automated solid-phase extraction in the 96-well format

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A sensitive and selective method was developed for the determination of (*R*)-ketoprofen ((*R*)-kt) and (*S*)-ketoprofen ((*S*)-kt) in human plasma using chiral liquid chromatography/tandem mass spectrometry (LC/MS/MS). Plasma samples spiked with stable-isotope-labeled [$^{13}C_1$, $^{2}H_3$]-(*R* and *S*)-ketoprofen, for use as the internal standards, were prepared for analysis using automated solid-phase extraction (SPE) in the 96-well microtiter format. The enantiomers were separated on an (*R*)-1-naphthylglycine and 3,5-dinitrobenzoic acid (Chirex 3005) 250 × 2.0 mm i.d. analytical column, equipped with a 30 × 2.0 mm i.d. guard column using isocratic mobile phase conditions. The (*R*)- and (*S*)-kt levels were quantifiable from 0.05 to 2500 ng ml^{-1} by constructing two separate curves from calibration standards covering the same range. The first curve ranged from 0.05 to 100 and the second from 100 to 2500 ng ml^{-1}. A concentration of 0.05 ng ml^{-1} of either enantiomer was easily detected using a 1 ml plasma sample volume. The average method accuracy, evaluated at four levels over an extended period, was better than ±3% over the entire range. The precision for the same set of quality control samples ranged from 4.0 to 7.0% RSD (*n* = 24). The method was applied to the evaluation of pharmacokinetic parameters in human plasma obtained from volunteers who received 25 mg of kt by peroral administration of Actron caplets or by topical administration of Oruvail gel.

KEYWORDS: ketoprofen; chiral liquid chromatography; tandem mass spectrometry; plasma; pharmacokinetics

INTRODUCTION

Ketoprofen, (*R*,*S*)-2-(3-benzoylphenyl)propionic acid, (kt) is a potent non-steroidal, anti-inflammatory drug possessing one chiral center that is currently marketed as the racemic mixture (Fig. 1). It is generally accepted that the (*S*) form contains the intrinsic pharmacological activity[1] and it is therefore desirable to perform enantiomer-selective concentration measurements in plasma. After peroral administration of a typical 25 mg dose, measurement of the levels of (*R*)- and (*S*)-kt is readily achieved by reported high-performance liquid chromatographic/ultraviolet (HPLC/UV) or gas chromatographic/mass spectrometric (GC/MS) methods over a significant time course; however, the circulating levels following topical administration are considerably lower and difficult to measure by either of these techniques. Although individual isomer levels following oral administration of ketoprofen have been reported, stereoselective measurement of plasma levels following topical administration has not.

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The literature is rich with analytical methods for the determination of ketoprofen and its enantiomers in biological matrices and pharmaceutical formulations. Some of the direct approaches for the analysis of ketoprofen and its enantiomers include HPLC with UV detection[2–9] and capillary zone electrophoresis with UV detection.[10,11] In addition, a number of indirect methods, whereby diastereomers (typically amides) were formed prior to non-chiral chromatography, have also been reported.[12–15] The lower limits of quantitation (LLOQ) achievable with these methods are typically in the 25–50 ng ml^{-1} range, which is not sufficient to define the pharmacokinetic (PK) profiles of plasma kt levels obtained after topical administration of a typical 25 mg dose. Furthermore, the chromatographic profiles obtained by these methods often contain extraneous matrix peaks complicating the analysis and, in general, these methods require 10–25 min for analysis.

Determination of ketoprofen has also been performed with MS-based techniques. Neat solutions of both enantiomers have been analyzed by LC/MS directly or following derivatization with benzofurazan reagents.[16] A bioanalytical assay for the purpose of screening for ketoprofen and other nonopiod analgesics has been performed with capillary electrophoresis/MS in human urine.[17] However, the majority of the bioanalytical analyses of ketoprofen by MS-based techniques have

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been performed using GC/MS.[18–22] Only one of these methods is enantiospecific; it is an indirect method requiring derivatization with subsequent separation of the diastereomers.[21] The LLOQs reported for the racemic GC/MS methods for the determination of ketoprofen in plasma range from 1 to 5 ng ml^{-1} while the LLOQ of the chiral method is 1 ng ml^{-1} for each enantiomer.

The study of the relative PK of both the (*R*) and (*S*) forms of kt in plasma after topical and oral administration requires a very sensitive analytical approach with a large dynamic range. This paper details the development, validation and application of a method involving stable-isotope dilution, chiral LC and electrospray ionization (ESI) MS/MS for the determination of (*R*)- and (*S*)-kt in human plasma. Plasma samples were spiked with [$^{13}C_1$, $^{2}H_3$]-(*R* and *S*)-ketoprofen (SIL (*R*)-kt and SIL (*S*)-kt, Fig. 1) for use as internal standards. The resulting mixture was prepared for analysis using automated solid-phase extraction (SPE) in the 96-well microtiter plate format. The plasma extracts were chromatographed on a chiral HPLC column with an (*R*)-1-naphthylglycine and 3,5-dinitrobenzoic acid chiral stationary phase. Following separation, the analytes and internal standards were selectively detected using ESI-MS/MS. The method has an LLOQ for (*R*)- and (*S*)-kt of 0.05 ng ml^{-1}, using a 1 ml plasma sample volume. The advantages of this methodology over previously published methods include an LLOQ (kt mL^{-1} plasma) that is approximately 500 times lower than that typically achieved using a chiral separation with UV detection and approximately 20 times lower than the chiral GC/MS approach. The LC/MS/MS method also utilizes automated sample preparation, does not require derivatization and has a relatively short run time of 6.5 min per sample for high throughput.

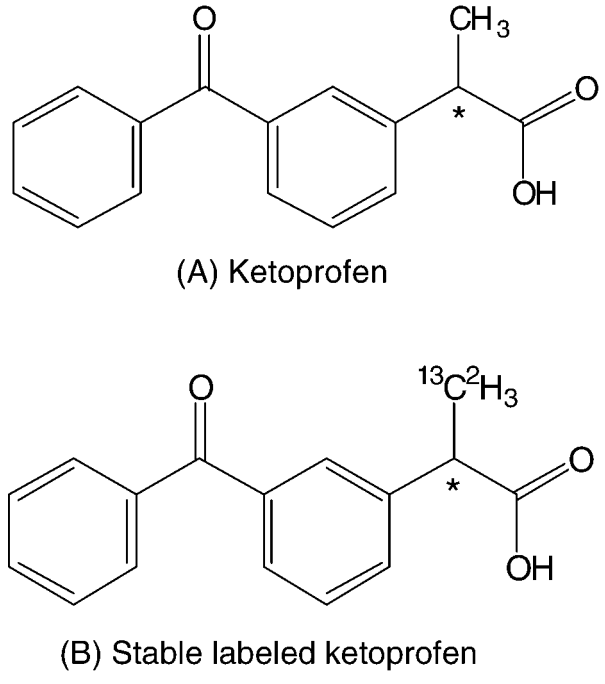

Figure 1. Structures of (A) (*R,S*)-ketoprofen and (B) [$^{13}C_1$, $^{2}H_3$]-(*R,S*)-ketoprofen. The asterisk denotes the chiral center.

EXPERIMENTAL

Chemicals and reagents

(*R,S*)-Ketoprofen was obtained from the United States Pharmacopeial Convention (Rockville, MD, USA) and [$^{13}C_1$,$^{2}H_3$]-(*R,S*)-ketoprofen was synthesized at P & G Pharmaceuticals, (Norwich, NY, USA). Optically pure (*S*)-kt was obtained from Aldrich (Milwaukee, WI, USA) and [^{3}H]-(*R,S*)-ketoprofen was obtained from American Radiolabeled Chemicals (St Louis, MO, USA). Methanol (HPLC grade), formic acid (reagent grade) and ammonium acetate for preparation of the HPLC mobile phase were purchased from J.T. Baker (Phillipsburg, NJ, USA). Formic acid (98%) for SPE was obtained from EM Science (Gibbstown, NJ, USA). Blank human plasma was obtained from volunteers at Procter & Gamble (Mason, OH, USA) or from Golden West Biologicals (Temecula, CA, USA); both sources of plasma used sodium heparin as the anticoagulant.

Preparation of standards

Calibration standards were prepared in plasma by adding known masses of (*R,S*)-ketoprofen to individual plasma aliquots, then processing in a fashion identical with that for the unknown test samples. Fifteen calibration standards were prepared and analyzed with each set of samples to cover a range of 5×10^4. The levels were 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng ml^{-1} of ketoprofen (as the racemate). These calibration standards were prepared on the day of analysis by adding either 10, 20 or 50 µl of a 20 : 80 methanol–water solution containing either 0.01, 0.10, 1.0, 10 or 100 µg kt ml^{-1} (again, as the racemate) to 1.0 ml of normal human plasma. The calibration standards were then processed by SPE, as described below.

Preparation of quality control samples

Quality control (QC) samples were prepared and analyzed with each sample set to monitor the system suitability and long-term method performance. These samples consisted of normal human plasma spiked with known quantities of (*R,S*)-ketoprofen. These QC samples were prepared by spiking a pool of blank human plasma with an appropriate mass to yield final plasma concentrations of 1.0, 10.0, 200 and either 1000 or 2000 ng ml^{-1} (as the racemate). Aliquots (1.0 ml) of the spiked samples were prepared for analysis by SPE, as described below. Typically three QCs at each level were analyzed with each set of unknown samples and were dispersed throughout the sequence by placing a set of QCs at each concentration at the beginning, middle and end of each sample set.

SPE procedure

Each calibration standard, QC sample or study sample was transferred to a designated position of a square well (2.0 ml) titer plate, using the Biomek 2000 automated workstation (Beckman Coulter, Fullerton, CA, USA). The

square well plate was then transferred to a Multimek 96-channel pipetter (Beckman Coulter), where 0.05 ml of formic acid and 0.025 ml of SIL kt solution (2 μg ml^{-1}) were sequentially added to each well containing a sample, calibration standard or QC sample.

SPE was performed using Oasis HLB sample extraction plates (Waters, Milford, MA, USA) with each column containing 30 mg of sorbent. The Biomek 2000 workstation was programmed to perform the column conditioning, sample loading, wash and elution steps automatically. The SPE columns were conditioned with 1.0 ml of water–methanol–formic acid (0 : 100 : 0.1), followed by 1.0 ml of water–methanol–formic acid (97 : 3 : 0.1). Following a mixing step, the entire sample, calibration standard or QC sample was then loaded on to the column. The columns were washed with 1.0 ml of water–methanol–formic acid (97 : 3 : 0.1), followed by 1.0 ml of water–methanol–formic acid (60 : 40 : 0.1). Finally, the analytes were eluted into HTS deep well tubes (Matrix Technologies, Lowell, MA, USA), using two 0.5 ml portions of water–methanol–formic acid (10 : 90 : 0.1). The solvent was removed from the tubes under a stream of nitrogen using a manifold with properly spaced drying needles and a heating block, both of which were designed and manufactured at the Procter & Gamble machine shop facility at Miami Valley Laboratories (Ross, OH, USA).

Prior to chromatographic analysis, each calibration standard, QC and unknown sample was reconstituted in 0.2 ml of methanol. Calibration standards and QC samples containing ≥20 ng ml^{-1} of kt were further diluted in methanol according to the following schedule: 20 and 50 ng ml^{-1} (1 : 5 dilution), 100 and 200 ng ml^{-1} (1 : 20 dilution), 500, 1000 and 2000 ng ml^{-1} (1 : 100 dilution) and 5000 ng ml^{-1} (1 : 200 dilution) prior to LC/MS/MS analysis. Plasma samples collected following a 25 mg oral dose of kt at 0.33 and 8.5 h after dosing were further diluted with methanol in a ratio of 1 : 20. Samples collected at 0.67 and 4.5 h post-dose were diluted 1 : 50 and samples collected at 1.33 and 2.50 h post-dose were diluted 1 : 100. All other standards, QCs and study samples were analyzed without further dilution.

Absolute recovery of ketoprofen from SPE

The recovery of ketoprofen from the Oasis HLB SPE columns was assessed using radiolabeled (*R*,*S*)-ketoprofen. Plasma was spiked to contain 8.4 ng ml^{-1} of the racemic mixture of [^{3}H]ketoprofen, equaling ~300 000 dpm ml^{-1} plasma sample. A 1 ml volume was loaded on to each of the 96 wells of a single extraction plate. All of the fractions were collected from all of the 96 columns, including the initial effluent, both of the wash steps and both of the 0.5 ml eluted fractions. All of the effluents were transferred to scintillation vials and counted using standard scintillation techniques to determine the activity, or mass of [^{3}H]ketoprofen, contained in each fraction. Based on the activity in the plasma, this procedure could accurately determine as little as 0.03% of the total mass contained in a single fraction.

Since the 96-well format places samples with varying concentrations in close proximity to each other, the potential for cross-contamination was evaluated. Again using [^{3}H]ketoprofen, normal human plasma was spiked to contain 24.5 ng ml^{-1} (~850 000 dpm ml^{-1} plasma). Using half of the 96-well plate, columns 7–12, samples were interspersed such that one well in each column was utilized. Plasma containing [^{3}H]ketoprofen (1.0 ml) was loaded on positions A7, D8, G9, B10, E11 and H12 and the SPE procedure was performed as described. The final elution fractions, 2 × 0.5 ml, from each row (A–H) of the six columns (7–12) were collected and counted using scintillation techniques. Considering the activity in each spiked plasma sample, as little as 0.01% of the total could be detected in any individual fraction.

LC/MS/MS conditions

A Gilson (Middletown, WI, USA) modular HPLC system consisting of a Model 305 control pump, two Model 306 auxiliary pumps, a Model 811C dynamic mixer, a Model 805 manometric module and a Model 234 autosampler comprised the chromatographic system. The enantiomers were separated on a Chirex 3005 stationary phase (Phenomenex, Torrance, CA, USA) consisting of an (*R*)-1-naphthylglycine and 3,5-dinitrobenzoic acid stationary phase (250 × 2.0 mm i.d.) which was preceded by a 30 × 2.0 mm i.d. guard column containing the identical sorbent. The mobile phase consisted of methanol–water (95 : 5) containing 30 mM ammonium acetate adjusted to pH 3.5 with formic acid. The flow-rate was 0.5 ml min^{-1}. Typically, 20 μl of the 0.2 ml reconstituted extract were injected onto the column, although a range of 10–50 μl was found to be acceptable. The entire chromatographic effluent was passed into the mass spectrometer interface for subsequent detection. Under these conditions, the HPLC retention times for (*R*)- and (*S*)-kt were ~4.7 and 5.1 min, respectively.

The mass spectrometer was a Perkin-Elmer SCIEX (Thornhill, Ontario, Canada) API III$^+$ operated in the TurboIonSpray configuration, consisting of the articulated IonSpray inlet used in conjunction with the heated TurboProbe desolvation unit. The TurboProbe temperature and nitrogen gas flow-rate were 450 °C and 8 l min^{-1}, respectively, and the nebulizer gas pressure was 60 psi (nitrogen). Protonated analyte ions were generated using ESI and orifice potentials of 3800 and 65 V, respectively. Collisional activation was achieved using argon as the collision gas at a thickness of 270 × 10^{13} molecules cm^{-2} and a collision energy of 17 eV. Resolution for both the precursor and product ions was opened until the peak width at half of the maximum intensity was ~1.2 Da. The selected reaction monitoring (SRM) transition *m*/*z* 255 to 209 was monitored for detection of (*R*)-kt and (*S*)-kt, while the SRM transition *m*/*z* 259 to 213 was monitored for SIL (*R*)-kt and SIL (*S*)-kt. The dwell time for each transition was 400 ms and the count control was set to a value of 1. Area ratios for the chromatographic peaks were determined using the Perkin-Elmer SCIEX software package MacQuan version 1.4.

Stability of kt in plasma

Aliquots of 4 ml of blank plasma were spiked to contain (*R*,*S*)-kt at the 5.0 ng ml^{-1} level. Four replicate aliquots (1.0 ml) of one sample were withdrawn, SIL kt was added and sample preparation was performed immediately. Four

additional aliquots were withdrawn and allowed to stand at room temperature for 4 h, prior to addition of SIL kt, and further processing. Additionally, four sample aliquots were subjected to three freeze–thaw cycles at −70 °C. Immediately following the third cycle, SIL kt was added, and sample processing commenced. The remainder of the kt spiked plasma was divided into 2.0 ml aliquots and stored frozen in cryovials at −70 °C. At 3 and 5 months after the initial stability studies had been conducted, two vials containing 2.0 ml of plasma were thawed and processed ($n = 4$) to assess long-term stability.

Human pharmacokinetic study

Six male volunteers received a 25 mg topical dose of ketoprofen in the form of Oruvail Gel. This product, which is commercially available in the UK, was applied on the bicep region of the left arm, and remained on the skin for the duration of the sampling period. Six additional volunteers received a 25 mg oral dose of ketoprofen, in the form of two 12.5 mg Actron caplets. For both treatment groups, venous blood (10 ml) was drawn prior to dosing, and at 20, 40 and 80 min and 2.5, 4.5, 8.5, 12.33, 16 and 24 h post-treatment. Plasma was collected and stored frozen at −70 °C in polypropylene cryovials until the time of analysis.

RESULTS AND DISCUSSION

Absolute recovery of ketoprofen from SPE

Using radiolabeled ketoprofen, it was determined that 60.1% of ketoprofen loaded on an Oasis column elutes in the first 0.5 ml fraction. An additional 14.9% is contained in the second 0.5 ml of eluate, yielding a combined sample extract containing 75% (±3.6%, $n = 96$) of the total mass loaded. Less than 3% of the total mass was contained in the remaining sample loading and wash step fractions, indicating that ~22% the total ketoprofen remains on the Oasis HLB sorbent, following the SPE procedure.

When [^{3}H]ketoprofen was loaded onto predetermined interspersed 96-well positions, fractions eluted from adjacent wells were monitored and no measurable activity was found in any of the effluents from column wells not containing a spiked sample. This indicates that the degree of contamination from one extraction column to another can generally expected to be ≤0.01% of the total mass of ketoprofen from any other 96-well plate position.

Chiral chromatography

By separating the enantiomers directly with a chiral stationary phase and using mass spectrometric detection, no lengthy derivatization procedures were needed to improve separation or sensitivity. The Chirex 3005 selector allowed the use of typical reversed-phase solvents that are conducive to ESI. Several other chiral stationary phases were evaluated including Chiralpak AD (Chiral Technologies, Exton, PA, USA) using normal-phase solvents. This column yielded better resolution of the enantiomers, but the use of normal-phase solvents was avoided because of the potential for inconsistent ion current and poorer sensitivity. Although others have circumvented these issues associated with use of normal-phase solvents, such as hexane, by using post-column mixing,[23,24] the mobile phase selected for use with the Chirex 3005 stationary phase was directly compatible with the ESI interface and did not require that additional experimental set-up.

The column configuration consisted of a 30 × 2.0 mm i.d. guard column followed by a 250 × 2.0 mm i.d. analytical column. This set-up provided a resolution factor (R_S) of 1.25. Other variations of column diameters and lengths were investigated to measure the effect on resolution of the enantiomers. A 250 × 4.0 mm i.d. column improved the resolution ($R_S = 2.22$) but increased the overall run time to near 10 min, even with correspondingly higher flow-rates. The larger diameter column also produced the expected loss in analyte concentration, thereby reducing the signal intensity by a factor of 3.5, as measured by signal-to-noise ratio. This decrease in sensitivity approaches the theoretical factor of 4 that is expected when column diameters are increased from 2.0 to 4.0 mm. The 250 × 2.0 mm i.d. column was selected for analysis because it was a practical compromise between resolution, analysis time and sensitivity.

Optically pure (*S*)-kt was obtained and chromatographed using the final conditions to determine the elution order of (*R*)- and (*S*)-kt. These experiments showed that the (*R*) form elutes first and (*S*)-kt elutes second. This elution order is consistent with previously published results using a Chirex 3005 stationary phase and a slightly differing mobile phase.[5]

ESI mass spectra

The ESI mass spectrum obtained for kt shows an abundant peak corresponding to the protonated molecular ion, $[M + H]^+$ at *m*/*z* 255, or ammonium adduct ion, $[M + NH_4]^+$, at *m*/*z* 272, depending on the choice of orifice potential. Analogous ions are produced from the stable-labeled material with a corresponding shift of 4 mass units. Product ion spectra for each type of precursor ion, either $[M + H]^+$ or $[M + NH_4]^+$, yield a prominent fragment ion at *m*/*z* 209. To determine which precursor/product ion transition provided superior sensitivity, the instrument conditions were optimized for each transition. A direct comparison was then performed by injecting a racemic mixture of ketoprofen spiked into plasma extracts using the optimized conditions for each SRM transition, and then measuring the response using the signal-to-noise ratio as the criterion. The results yielded a virtually identical response for each set of transitions. The $[M + H]^+$ ion was chosen as the precursor only because the chemical noise was marginally lower. The product ion spectra obtained following collisional activation of the protonated molecular ion is shown in Fig. 2 for (*R*,*S*)-ketoprofen and its SIL kt counterpart, [$^{13}C_1$, $^2H_3$]-(*R*,*S*)-ketoprofen. The SRM transition of *m*/*z* 255 to 209 was chosen for (*R*)- and (*S*)-kt, and *m*/*z* 259 to 213 was used for SIL (*R*)- and SIL (*S*)-kt for quantitative method development.

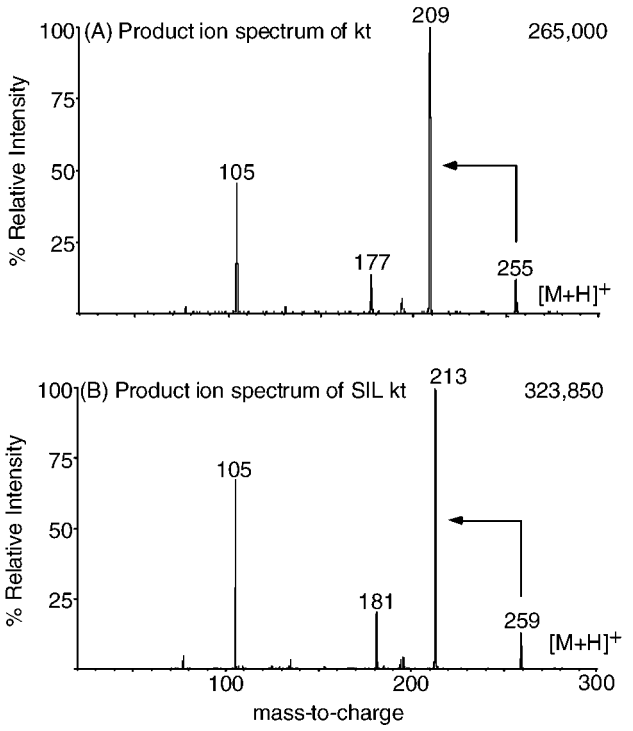

Figure 2. Electrospray ionization product ion spectra of (A) (*R,S*)-ketoprofen and (B) [$^{13}C_1$, $^{2}H_3$]-(*R,S*)-ketoprofen. The arrows indicate the transitions used for quantitation.

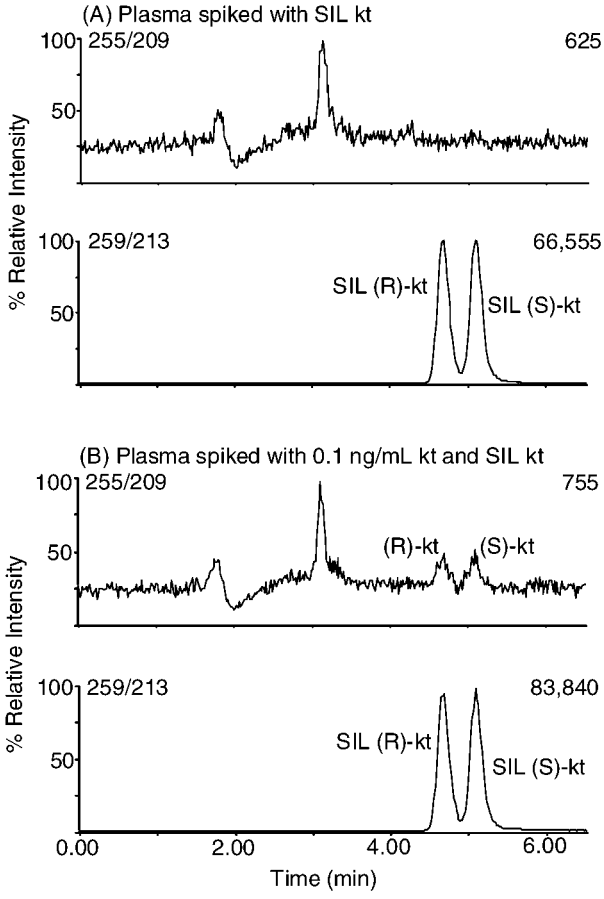

Figure 3. LC/MS/MS chromatographic profiles for kt and SIL kt generated during the analysis of (A) a 1 ml human plasma sample spiked with 50 ng ml^{-1} of SIL kt and (B) a 1 ml calibration standard containing 0.1 ng ml^{-1} (*R,S*)-ketoprofen and 50 ng ml^{-1} SIL kt. The peaks shown for (*R*)- and (*S*)-kt represent 0.05 ng ml^{-1} (~0.01 ng on-column) of each enantiomer.

LC/MS/MS chromatographic profiles of plasma extracts

A typical chromatogram of a normal human plasma sample spiked only with SIL kt is shown in Fig. 3(A), and a calibration standard containing 0.1 ng ml^{-1} of ketoprofen as the racemic mixture is depicted in Fig. 3(B). The SIL kt was checked for possible contamination with unlabeled kt and the level of contamination was measured as <0.01%; it did not interfere with quantitation at the kt concentrations used in these studies. The average recoveries of the lowest calibration standard for both (*R*)- and (*S*)-kt obtained during eight batches of method application were 97.9% (3.5% RSD) and 98.0% (6.7% RSD), respectively.

Two examples of the chromatographic profiles generated during the analysis of study samples are displayed in Fig. 4. The plasma sample used to generate Fig. 4(A) was obtained from a patient who had received a 25 mg topical dose of ketoprofen and whose plasma was collected at 4.5 h post-dose. This plasma sample was found to contain 1.49 ng ml^{-1} (*R*)-kt and 1.19 ng ml^{-1} (*S*)-kt. Figure 4(B) was generated from a 24 h plasma sample obtained from a patient who had received a 25 mg oral dose of ketoprofen. This plasma sample contained 1.79 ng ml^{-1} (*R*)-kt and 2.70 ng ml^{-1} (*S*)-kt.

Quantitation of (*R*)- and (*S*)-kt

The ketoprofen calibration standards were prepared using the racemic mixture. The exact mass of each individual enantiomer contained in each calibration standard was estimated prior to the construction of the calibration curves. Since the enantiomers were not completely baseline resolved, integrated peak areas were determined by first dropping a vertical line from the valley of the two peaks to the baseline. The percentage of each enantiomer could then be calculated by dividing the absolute integrated peak areas of either (*R*)- or (*S*)-kt for selected calibration standards by the combined areas of both enantiomers and multiplying by 100. The final percentages were obtained from the average of several calibration standards. The percentage values obtained in this manner were used to compute the exact concentration of either (*R*)- or (*S*)-kt in each calibration standard. Performing this exercise always rendered (*S*)-kt level slightly higher than (*R*)-kt, with the percentage of (*S*)-kt ranging from 50.5 to 53.0% of the mixture.

Calibration curves for (*R*)-kt were constructed by plotting peak area ratios ((*R*)-kt/SIL (*R*)-kt) of standards versus (*R*)-kt concentration and fitting these data to a weighted ($1/x^2$) regression line. Drug concentrations in unknown samples were then interpolated from this line. The concentrations of (*S*)-kt in plasma were determined analogously.

Calibration standards were analyzed over a wide range from 0.05 to 2500 ng ml^{-1} for each enantiomer, representing nearly five orders of magnitude, so that full PK curves for both the topical and oral samples could be determined under one set of instrumental conditions.

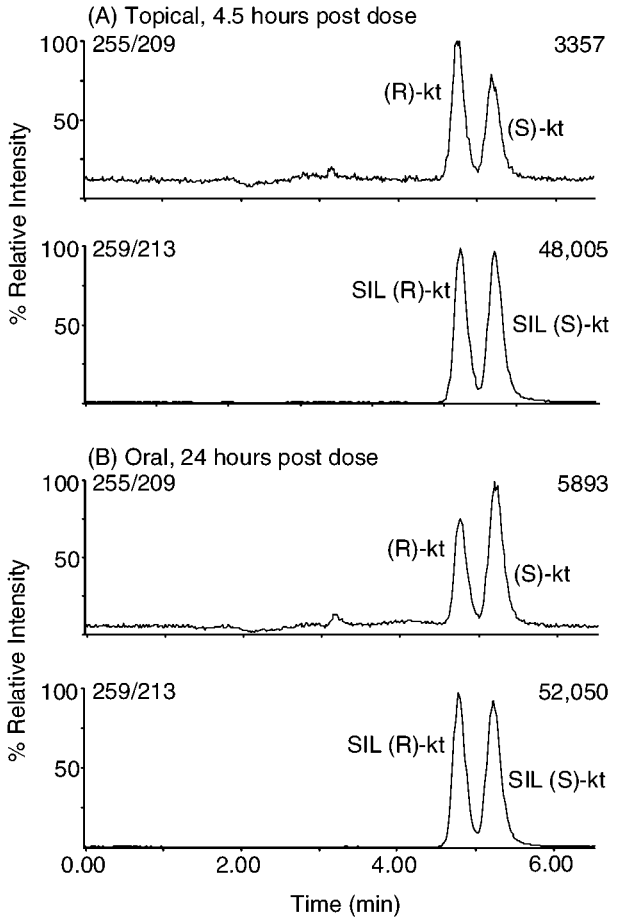

Figure 4. LC/MS/MS chromatographic profiles for kt and SIL kt generated during the analysis of (A) a 1 ml study sample collected 4.5 h following a 25 mg topical dose of (*R,S*)-ketoprofen and (B) a 1 ml study sample collected 24 h following a 25 mg oral dose of (*R,S*)-ketoprofen.

In order to avoid signal saturation due to instrument limitations, and to preserve the linearity of response, it was necessary to dilute the higher calibration standards, QCs and study samples. This dictated the necessity to balance the mass of internal standard added to each calibration standard such that when undiluted, instrument saturation did not occur, while still containing enough mass to yield a quantifiable peak area when diluted 200-fold. The internal standard level of 50 ng, as the racemate, met both requirements.

Although a single, weighted calibration curve covering the entire calibration standard range provided acceptable linearity, method accuracy was improved by segmenting the calibration curve into a low and a high portion. One curve was constructed using calibration standards from 0.05 to 100 ng ml^{-1}, while the second segment ranged from 100 to 2500 ng ml^{-1}. Typical equations of the lines used for the lower and upper ranges of (*R*)-kt calibration are $y = 0.0321x + 0.000334$ and $y = 0.0316x - 0.124$, respectively. Typical equations of the lines used for (*S*)-kt quantitation are $y = 0.0285x - 0.000023$ and $y = 0.0307x - 0.394$ for the lower an upper ranges, respectively. Average correlation coefficients for calibration curves used during eight batches of method application were 0.997 and 0.998 for the lower and upper (*R*)-kt curves and 0.998 for both (*S*)-kt calibration curves.

Accuracy and precision—validation and QC samples

The accuracy and precision data for the LC/MS/MS analysis of blank human plasma spiked with (*R*)- and (*S*)-kt at various levels are presented in Tables 1 and 2. Table 1 contains data that were collected during method validation. Table 2 summarizes the QC samples that were prepared and analyzed with samples from two PK studies over a 4 month period. As can be seen from these data, the recoveries of the QCs during clinical study analysis are more accurate and precise than the validation QCs. Initial application of the methodology showed that, occasionally, blank samples contained a slight response for kt that was not observed upon re-preparation of that sample. Therefore, two subtle changes in the methodology that were introduced between the original validation and the long-term application are as follows: (1) contents of the square well plates were mixed with the Biomek 2000 by pipetting and dispensing the contents rather than with a tube vortexer and (2) samples, standards or QCs with kt concentrations >200 ng ml^{-1} were placed adjacent to empty wells or other samples with kt concentrations >200 ng ml^{-1}. With these additional criteria for preparation, the average long-term accuracy improved to within ±3% with RSDs of 7% or better and there was no longer a problem with contamination of blank samples.

Stability of kt in plasma

The stability of (*R*)- and (*S*)-kt in the plasma matrix was examined by analyzing plasma samples spiked with

Table 1. Method validation spike and recovery results for (*R*)- and (*S*)-kt in human plasma (*n* = 11, unless noted otherwise)

Analyte	Concentration (ng ml^{-1})	Mean recovery (%)	RSD (%)
(*R*)-kt	1000	100.2 ($n = 8$)	9.1
	500	109.3 ($n = 3$)	3.1
	100	100.0	5.8
	5.0	105.2	13.7
	0.5	103.4	10.0
(*S*)-kt	1000	105.0 ($n = 8$)	8.0
	500	107.7 ($n = 3$)	5.2
	100	98.1	6.7
	5.0	100.4	15.2
	0.5	102.6	6.0

Table 2. Average (*R*)- and (*S*)-kt QC recoveries obtained during the analysis of eight batches of clinical samples from two PK studies over a 4 month period (*n* = 24)

Analyte	Concentration (ng ml^{-1})	Mean recovery (%)	RSD (%)
(*R*)-kt	500	102.1	7.0
	100	97.5	6.2
	5.0	99.7	4.8
	0.5	102.8	4.0
(*S*)-kt	500	101.1	5.6
	100	100.0	6.3
	5.0	100.7	4.8
	0.5	101.1	5.0

(*R*,*S*)-kt (5.0 ng ml^{-1}). The recovery for plasma samples ($n = 4$) prepared immediately after spiking was 101.0% (4.2%) and 100.0% (3.1%) for (*R*)- and (*S*)-kt, respectively, with the RSDs in parentheses. After 4 h of standing at room temperature, the recoveries were 100.1% (2.6%) and 98.7% (1.8%) for (*R*)- and (*S*)-kt, respectively, and following three freeze–thaw cycles, recoveries of 98.1% (1.9%) and 98.1% (2.4%) were obtained. Long-term storage of plasma spiked with kt at −70 °C was also investigated. After 3 months of frozen storage, the recoveries of (*R*)- and (*S*)-kt using a freshly prepared calibration curve were 93.3% (2.7%) for (*R*)-kt and 94.2% (3.0%) for (*S*)-kt and after 5 months the recoveries were 93.7% (1.7%) and 93.2% (2.4%), respectively. For plasma stored up to 4 h on the bench, exposed to three cycles of freezing and thawing or stored up to 5 months at −70 °C, the kt recovery was within 10% of the value obtained with freshly spiked plasma.

The stability of the acyl glucuronide, the major metabolite of kt, is of concern for sample preparation of plasma samples obtained from volunteers dosed with kt.[4] To minimize the chance of degradation of the acyl glucuronide to ketoprofen, plasma samples were quickly frozen at −70 °C and were acidified prior to sample preparation. Chiral inversion of (*R*)- and/or (*S*)-kt during sample preparation was also investigated. The ratio of (*R*)- to (*S*)-enantiomers was measured for a racemic mixture prepared as a neat solution in methanol–water (20 : 80) and compared with the ratio obtained by spiking kt into plasma and completing the SPE preparation procedure. For $n = 10$ neat samples, the amount of (*R*)-kt was found to be 48.8% (RSD 1.1%) and that of (*S*)-kt was 51.2% (1.0%). For the samples spiked into plasma and taken through the preparation procedure, the amount of (*R*)-kt was found to be 49.2% (1.2%) and that of (*S*)-kt was 50.8% (1.2%). No significant bias was introduced by spiking the sample into plasma or by performing the sample preparation procedure.

Human pharmacokinetic profiles

Plots of (*R*)- and (*S*)-kt levels in human plasma versus post-treatment time, following a 25 mg kt dose from commercially available topical (Oruvail) and oral (Actron) formulations, are shown in Fig. 5. The sensitivity of the LC/MS/MS methodology facilitated the measurement of (*R*)- and (*S*)-kt levels following the oral route of administration, in every post-dose sample, in all subjects up to 24 h after drug delivery. In subjects receiving the topical dose, measurable drug levels typically first appeared at 2.5 h post-dose, although one subjects' plasma did contain measurable drug as early as 1.33 h, while another subject required 4.5 h to observe drug. Following the onset of measurable drug levels with the topical treatment, (*R*)- and (*S*)-kt were detected in all remaining samples until the end of the 24 h time course. The 24 h sample accounted for the maximum concentration in all subjects, following topical treatment. For the 24 h time course followed in this study, it is apparent that the area under the PK curve generated with the topical formulation is much lower than that observed with the oral formulation.

Higher plasma concentrations of (*S*)-kt relative to (*R*)-kt were observed at the later time points of the oral PK curves. The observation of the enantiomeric excess of (*S*)-kt at later time points following an oral dose was consistent with previously reported results.[4] A slight preference was noted for the (*R*)-enantiomer at the early time points after topical administration as shown in Figs 4(A) and 5, which may indicate that the topical absorption process is somewhat enantioselective. The average ratio of (*S*)-kt to (*R*)-kt then slowly increased over the time course studied,

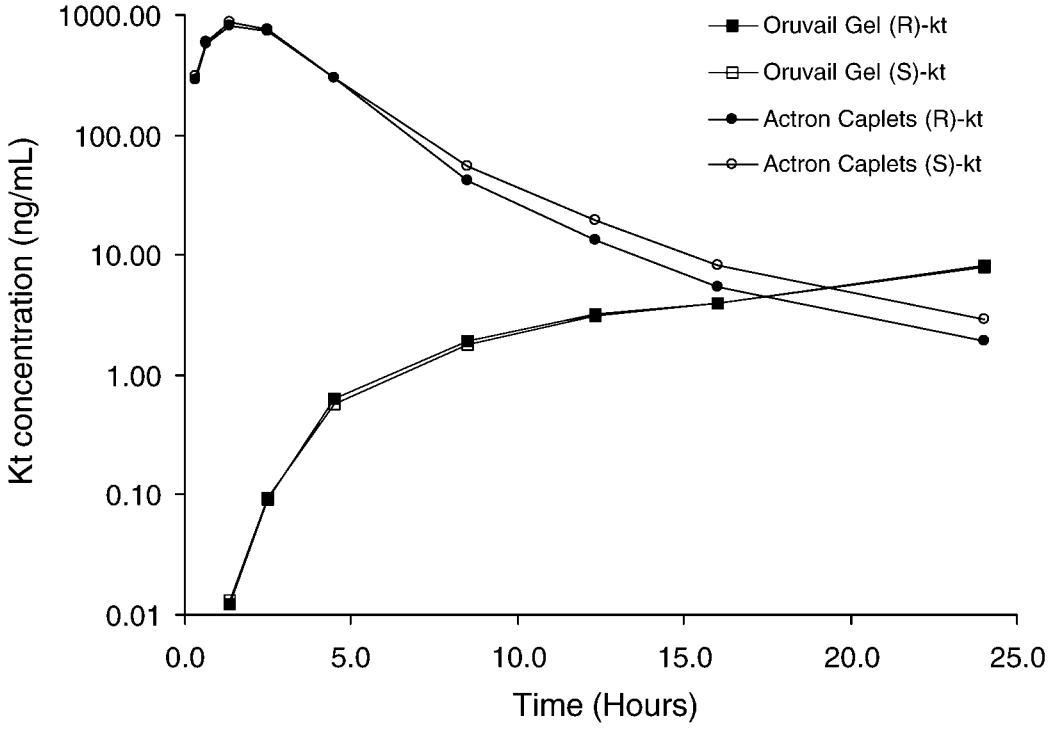

Figure 5. Human plasma levels of (*R*)- and (*S*)-kt following either a 25 mg peroral dose of kt as Actron caplets or a 25 mg topical dose of kt in the form of Oruvail Gel. Each point represents the mean of six subjects.

as might be expected given the oral results; however, kt was continually introduced into the blood throughout the 24 h time course, as is reflected by the increase in plasma concentrations. This caused the ratio of (*S*)-kt to (*R*)-kt to increase more slowly than was observed with the oral dose form. Given that information, increasingly higher levels of (*S*)-kt relative to (*R*)-kt might be expected as the total kt concentration in plasma decreases at time points later than the 24 h data presented here.

CONCLUSIONS

The combination of techniques employed, including solid-phase extraction, stable-isotope dilution, chiral LC and MS/MS, resulted in a rugged, sensitive, accurate and precise method for the determination of (*R*)- and (*S*)-kt enantiomers in human plasma at levels from 0.05 to 2500 ng ml^{-1}. Using this methodology, an extraction plate containing 96 samples, calibration standards and/or QC samples can easily be prepared in less than 1 h and then be analyzed by LC/MS/MS in less than 10.5 h, with a 6.5 min injection-to-injection analysis time. Multiple plate batches were also analyzed and, to date, the methodology has been successfully utilized to support two clinical studies involving several hundred plasma samples. The method has the required sensitivity to measure drug levels at 24 h following a 25 mg oral dose, and to quantitate kt in plasma following a 25 mg topical dose beginning at 2.5 h post treatment.

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