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# DEDERMINATION OF KETOPROFEN LEVELS IN HUMAN PLASMA BY FULLY VALIDATED HPLC ASSAY

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**ABSTRACT:**

We developed and validated a sensitive, specific, and rapid HPLC assay for ketoprofen measurement in human plasma and studied its stability under various conditions. After extraction with the *tert* butyl methyl ether, ketoprofen and diclofenac (internal standard) were eluted on 8 Nova-Pak $C_{18}$, 4 µm cartridge at room temperature (RT) with a run time of 10 min. The mobile phase consisted of 0.2% glacial acetic acid (pH 3.0) and acetonitrile (50:50, v:v). The analytes were detected using photodiode array detector set at 258 nm. The response was linear over the range of 0.02–10 µg/ml. Extraction recovery of ketoprofen and inter-run bias and precision were ≥ 90% (mean 96%), ≤ 2%, and ≤ 8.1%, respectively. Ketoprofen was stable in plasma for 24 hours at RT (100%), 10 weeks at –20 $^0$C (≥ 97%), and after 3 cycles of freeze at –20$^0$C and thaw at RT (98%). In extracted samples, ketoprofen was stable for 24 hours at RT (≥ 94%) and 48 hours at–20$^0$C (99%). Stock solution of ketoprofen (1 mg/ml in methanol) was stable for 48 hours at RT (100%) and 10 weeks at –20$^0$C (96%). Ketoprofen level was 3.64 µg/ml one hour after the ingestion of a 50 mg therapeutic dose. The data indicate that the described assay is suitable for ketoprofen therapeutic drug monitoring and bioequivalence studies in humans.

**Key words:** Diclofenac, HPLC, Ketoprofen, Stability, Validation.

## INTRODUCTION

Ketoprofen, 3-benzoyl-α-methyl (CAS number: 22071-15-4), is a propionic acid derivative with well recognized analgesic, anti-inflammatory, antipyretic, and antirheumatic properties Ketoprofen exerts inhibitory effects on

prostaglandin and leukotriene synthesis, antibradykinin effects, and lysosomal membrane stabilizing activity[1,2].

Ketoprofen is rapidly and efficiently absorbed from the gastrointestinal tract, extensively bound to plasma proteins, has two inactive metabolites produced through liver metabolism, and has a plasma half-life of approximately 2- 4 h (http://www.micromedex.com/products/drugdex/). After oral administration of 50 or 200 mg ketoprofen, peak serum concentrations were 2 - 3.9 µg/ml and 3.1 - 3.4 µg/ml, respectively (http://www.micromedex.com/products/drugdex/).

Although, several methods[3-25] have been described for the determination of ketoprofen level in biological samples; They are limited by low sensitivity[5,6,9-11,14,15,18,19], or low recovery[11,14], require laborious preparation[22-25], long run times[6,7,14], or equipments that are not readily available; or have not been validated in human plasma[4,7,8,10,13,17,18,20,22]. Further, limited information is available on the stability of ketoprofen[3, 5-9, 11, 13-18, 20, 23].

The aims of this study were to 1) develop and fully validate a sensitive, specific, and rapid HPLC assay to measure ketoprofen level in human plasma, and 2) determine the stability of ketoprofen under various clinical laboratory conditions.

**Materials and methods**

**Apparatus:**

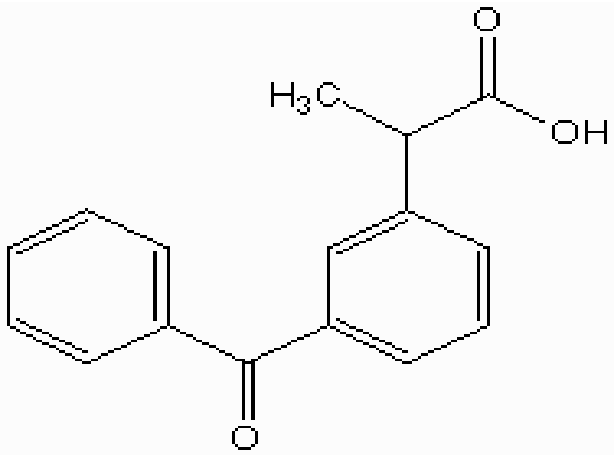
The liquid chromatograph consisted of Waters Alliance 2690 Separations Module, an 8 x 100 mm, 8 Nova-Pak $C_{18}$, 4 µm (particle-size) cartridge column, a Guard Pak pre-column module with Nova-Pak C18, 4 µm insert, and Waters 996 photodiode array detector (Water Associates, Milford, MA, USA) set at 258 nm. Data were collected with a Pentium III computer using Millennium[32] Chromatography Manager Software (Water Associates, Milford, MA, USA).

**Chemicals and reagents:**

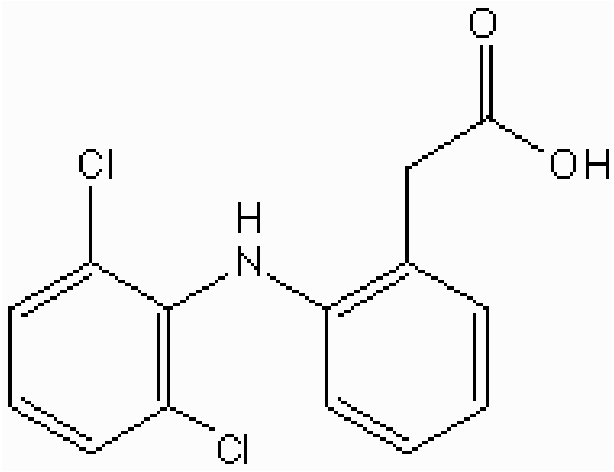
Ketoprofen standard (Figure 1-a) was purchased from Sigma-Aldrich, St. Louis, MO, USA, and diclofenac sodium standard (CAS number; 15307-86-5) (Figure 1-b) from USP, Rockville MD, USA. Acetonitrile, and glacial acetic acid (HPLC grade) were purchased from Fisher Scientific, Fairlawn, NJ, USA. *Tert.* butyl methyl

ether (HPLC grade) was purchased from Fluka Chemie AG, Switzerland. Water for HPLC was prepared by reverse osmosis and further purified by passing through a Millipore-Synergy UV (Millipore Co., Bedford, MA, USA).

**Figure-1: Chemical structures of ketoprofen (a) and diclofenac (b).**




**(a)**




**(b)**

**Figure-1**

**Chromatographic conditions:**

The mobile phase consisted of 0.2% glacial acetic acid (pH 3.0) and acetonitrile (50:50, v:v). It was filtered through a 0.45 µm size membrane filter (Millipore Co., Bedford, MA, USA), degassed, and delivered at 2 ml/min.

**Preparation of stock and working solutions:**

Ketoprofen stock solution (1 mg/ml) was prepared in methanol and used for stability studies and to prepare a working solution (20 µg/ml) in plasma. The working solution was prepared weekly to construct calibration curve and quality control (QC) samples. The internal standard (diclofenac) working solution (100 µg/ml) was prepared weekly in mobile phase from a stock solution (1 mg/ml) in methanol.

**Calibration standard / Quality control samples:**

Calibration standards were prepared by mixing appropriate volumes of ketoprofen working solutions with blank human plasma to produce final concentrations of blank, zero (blank plasma spiked with IS only), 0.02, 0.04, 0.08, 0.16, 0.5, 1.5, 4, 8, and 10 µg/ml. QC samples were prepared by mixing appropriate volumes of ketoprofen working solution in blank human plasma to produce final concentrations of 0.02, 0.06, 5, and 9 µg/ml. Samples were vortexed for 20 s, and 1 ml aliquots were transferred into Teflon-lined, screw-capped, borosilicate glass, 13 x 100 mm culture tubes (Fisher Scientific Co., Fairlawn, NJ, USA), and stored at –20ºC.

**Sample Preparation:**

Aliquots of 1 ml of calibration standards or QC samples were allowed to equilibrate to room temperature. To each tube, 200 µl of the 100 µg/ml internal standard (IS) working solution was added and vortexed for 10 s. After the addition of 6.0 ml of *tert* butyl methyl ether, the mixture was vortexed again for 5 min and then centrifuged for 15 min at 13200 rpm at room temperature. The organic layer was carefully collected, dried under a gentle stream of nitrogen at 40ºC, and the residue was reconstituted in 250 µl mobile phase and centrifuged at 3500 rpm for 3 min. The supernatant layer was carefully transferred into the auto-sampler vials and 100 µl were injected into the HPLC system. The run time was 10 min.

**Stability studies:**

Stability of ketoprofen in plasma: Adequate numbers of aliquots of two QC samples (0.06, and 9 µg/ml) were prepared. Aliquots were analyzed in 5 replicates immediately (baseline), after being processed and stored at room temperature for 24 h or at $-20^0$C for 48 h (auto-sampler stability), after being allowed to stand on the bench-top

for 8 or 24 h at room temperature before processing (counter stability), after being stored at –20$^0$C for 10 weeks before processing (long term freezer stability), or after being repeatedly stored at –20$^0$C for 24 h and then left to completely thaw unassisted at room temperature before processing.

Stock solutions stability: Five aliquots of the stock solutions of ketoprofen and the IS were analyzed (after dilution to 10 µg/ml in mobile phase) at baseline, after storage for 48 h at room temperature, or after storage at –20$^0$C for 10 weeks. Stability of the working solutions of ketoprofen and the IS, were evaluated up to 2 weeks at –20$^0$C.

**Assay validation method:**

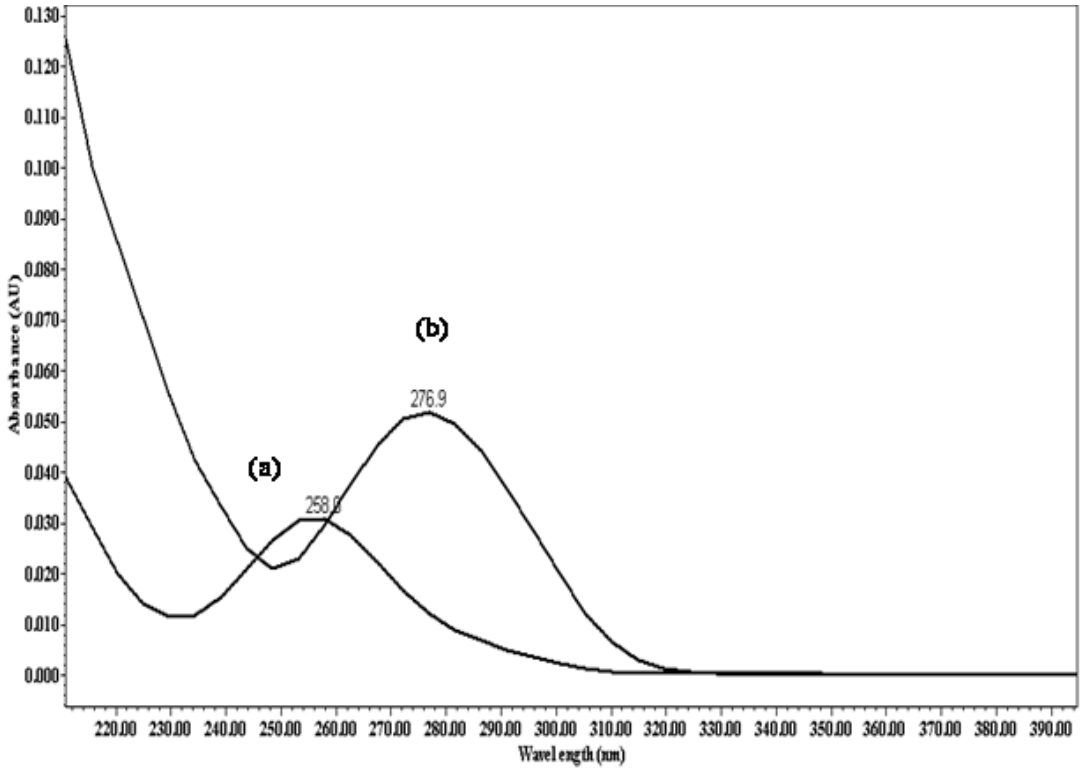
The presented method was validated according to FDA guidelines (http://www.fda.gov/cvm) in terms of intra-run and inter-run precision and accuracy, linearity, specificity, recovery, and stability.

**RESULTS**

**Optimization of chromatographic conditions:**

During method development, different combinations of the components of the mobile phase, different pH, and different flow rates were investigated to optimize separation of ketoprofen and the IS. A mobile phase composed of 0.2 % glacial acetic acid (pH 3.0) and acetonitrile (50:50, v:v) was found best to achieve adequate separation, minimize background absorbance, and avoid peak tailing. In order to improve specificity and detection limit, and minimize interference from plasma or solvent system that may occur at lower wavelengths, we searched for the best absorbance wavelength of ketoprofen in the concentration range studied using photodiode array extracted spectra (Figure 2); and preformed the analysis at 258 nm was found to be the optimum. In regard to, sample preparation, the best results were obtained when the ratio of plasma to *tert* butyl methyl ether was 1:6 (v:v). We used a Guard Pak pre-column module with Nova-Pak $C_{18}$, $_4$ µm insert to reduce damage to the analytical column. Under the described conditions, ketoprofen and the IS were resolved within a run time of 10 min, with a retention time of 4.1 and 8.7 min, respectively.

**Figure-2: PDA extracted ultraviolet spectra of ketoprofen (a) and diclofenac (b).**




**Linearity:**

Linearity was determined in the range of 0.02 – 10 µg/ml using ten calibration curves. The data were analyzed by using linear regression: *Conc. = a + b (PAR)*, where *Conc*. is the concentration of ketoprofen, *a* is the intercept, *b* is the slope, and *PAR* is the peak area ratio of ketoprofen divided by the peak area of the IS. The concentrations of the calibration standards of the ten calibration curves were back-calculated using the individual regression lines. Linearity studies (n=10) showed mean (SD) for $R^2$ of 0.9996 (0.0005), slope of 2.9749 (0.0401), and intercept of 0.0011 (0.0022). Figure 3 depicts an overlay of chromatograms of a representative standard curve. Figure 4 shows an overlay of two chromatograms of plasma samples collected from a healthy volunteer before and 1 h after the oral administration of a single 50 mg regular-release ketoprofen tablet.

**Figure-3: Overlay of ketoprofen calibration curve chromatograms spiked with diclofenac. The insert is a blow up of the lower concentrations.**

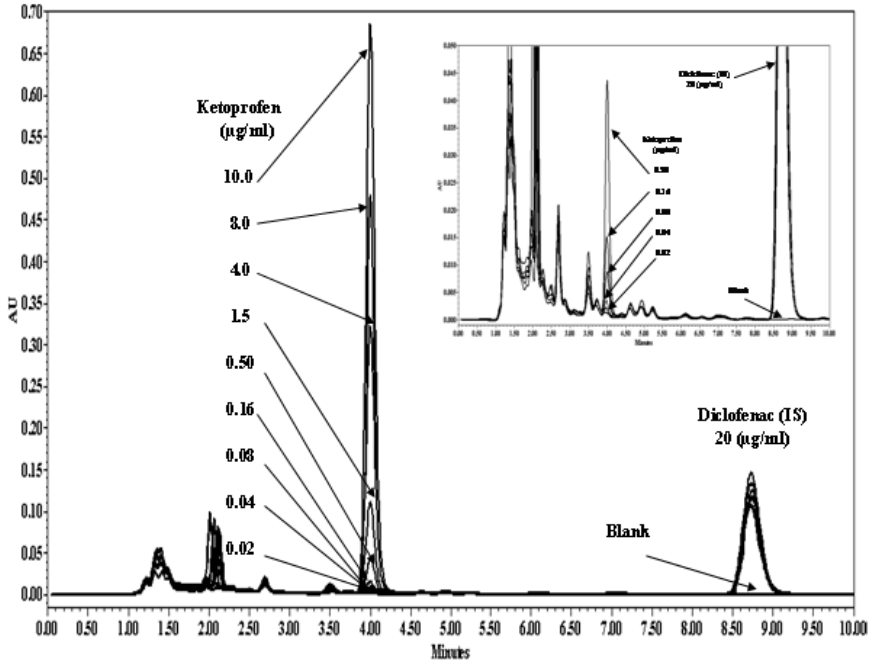
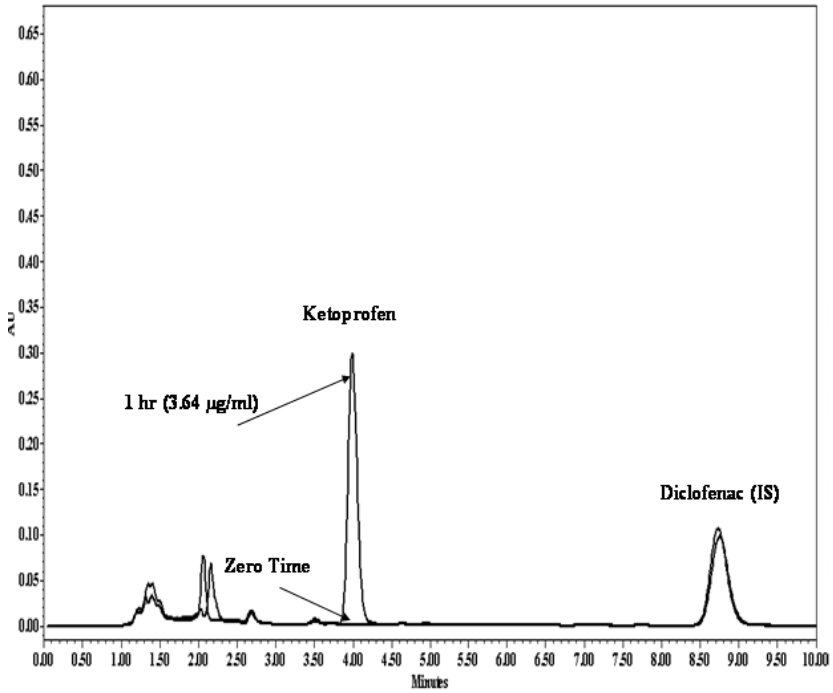



**Figure-4: Overlay of two chromatograms of volunteer plasma samples before and 1 hour after oral administration of a single 50 mg regular release ketoprofen tablet.**



**Limit of detection:**

The limit of detection (LOD), defined as three times the baseline noise, was 0.01 µg/ml.

**Specificity:**

To evaluate specificity, we screened seven frequently used medications and six different batches of human plasma. All batches of blank plasma were free from interfering components. None of seven commonly used drugs co-eluted with ketoprofen or the IS (Table 1).

**Table-1: Specificity of Ketoprofen Assay.**

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| Drug name | Retention time |
|---|---|
| Ketoprofen | 4.1 |
| Diclofenac sodium (IS) | 8.7 |
| Aspirine | 1.5 |
| Acetaminophen | 1.2 |
| Ranitidine | 3.5 |
| Nicotinic Acid* | 1.7 |
| Ascorbic Acid* | 1.5 |
| Caffeine* | 1.8 |
| Omeprazole | 3.1 |

Drug solutions (1 mg/ml) in methanol or water * were diluted to (10 µg/ml) in mobile phase and 100 µl were injected.

**Recovery:**

The extraction recovery of ketoprofen was determined by dividing mean peak areas of five replicates of four quality control samples (0.02, 0.06, 5, and 9 µg/ml) prepared in plasma (as described under sample preparation), by mean peak areas of five replicates of equivalent concentrations prepared in the mobile phase. The recovery of the IS was determined similarly at a concentration of 20 µg/ml. The results of the extraction recovery studies of

ketoprofen and the IS are presented in Table 2. Recovery was ≥ 90% (mean 96%) for ketoprofen and 87% for the IS.

**Table-2: Extraction Recovery of Ketoprofen and Ibuprofen.**

| Nominal Concentration (µg/ml) | Plasma | | Mobile Phase | | ** Recovery (%) |
|---|---|---|---|---|---|
| | *Mean Peak Area | SD | *Mean Peak Area | SD | |
| **Ketoprofen** | | | | | |
| 0.02 | 13258 | 550 | 14708 | 857 | 90 |
| 0.06 | 32939 | 445 | 33383 | 211 | 99 |
| 5 | 2447197 | 198833 | 2511413 | 3481 | 97 |
| 9 | 4391679 | 856608 | 4530013 | 2776 | 97 |
| **Diclofenac (IS)** | | | | | |
| 20 | 1630121 | 127853 | 1875505 | 68382 | 87 |

* Mean peak area of 5 replicates. ** Mean peak area of spiked plasma sample divided by mean peak area of spiked mobile phase sample x 100. SD, Standard Deviation.

**Precision and bias:**

Precision was calculated as coefficient of variation (standard deviation divided by mean measured concentration x 100), and bias as the absolute value of (1 minus mean measured concentration divided by nominal concentration) x 100. The intra-run and inter-run precision and bias of ketoprofen were determined by analyzing four QC samples: 0.02, 0.06, 5, and 9 µg/ml over three days (Table 3). Intra-run precision and bias (n = 10) ranged from 2.9 % to 5.7 % and from <1 % to 6 %, respectively. The inter-run precision and bias (n = 20) ranged from 3.4% to 8.1% and from 1 % to 2 %, respectively.

**Table-3: Intra-run and Inter-run Accuracy and Precision of Ketoprofen Assay.**

| Nominal Concentration (µg/ml) | Intra-run (n=10) | | | | Inter-run (n=20) | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean Measured Concentration (µg/ml) | SD | Precision (CV*, %) | ** Bias (%) | Mean Measured Concentration (µg/ml) | SD | Precision (CV*, %) | ** Bias (%) |
| 0.02 | 0.02 | 0.001 | 5.7 | 6 | 0.02 | 0.002 | 8.1 | 2 |
| 0.06 | 0.06 | 0.003 | 4.3 | 4 | 0.06 | 0.003 | 4.0 | 2 |
| 5 | 4.9 | 0.141 | 2.9 | 3 | 4.90 | 0.169 | 3.4 | 2 |
| 9 | 9.04 | 0.390 | 4.3 | <1 | 8.94 | 0.351 | 3.9 | 1 |

* Coefficient of variation (CV) = Standard Deviation (SD) divided by mean measured concentration x 100. **Bias = absolute value of 1 minus mean measured concentration, divided by nominal concentration x 100.

**Stability:**

The stability of ketoprofen under usual laboratory storage conditions of plasma and extracted samples was investigated. The results are presented in Table 4. The data indicate that ketoprofen is stable: 1) in plasma, for at least 24 h at room temperature or 10 weeks at –20$^0$C, and after at least three cycles of freeze at –20$^0$C and thaw at room temperature, 2) in extracted samples, for at least 24 h at room temperature or 48 h at –20$^0$C, and 3) in methanol (1 mg/ml) for at least 48 h at room temperature or 10 weeks at –20$^0$C. The IS stock solution (1 mg/ml) in methanol was also stable under the same conditions (96 % and 94 %, respectively). Further, the working solutions of ketoprofen (20 µg/ml in plasma) and the IS (100 µg/ml in mobile phase) were stable for at least 2 weeks at –20$^0$C (106 % and 96 %, respectively).

**Table-4: Stability of Ketoprofen in Plasma Samples and Stock Solution.**

| Nominal Concentration (µg/ml) | *Plasma Samples | | | | | | | | **Stock solution | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Unextracted | | | Extracted | | Freeze-thaw | | | | |
| | 8 h RT | 24 h RT | 10 wk –20$^0$C | 24 h RT | 48 h –20$^0$C | One cycle | Two cycles | Three cycles | 48 h RT | 10 wk –20$^0$C |
| 0.06 | 98 | 100 | 100 | 97 | 99 | 93 | 99 | 98 | 100 | 96 |
| 9 | 100 | 100 | 97 | 94 | 99 | 100 | 100 | 98 | | |

Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. ***** Spiked plasma samples were extracted and analyzed immediately (baseline, data not shown), after 8 or 24 hours at room temperature (8 h RT and 24 h RT), after 10 weeks at – 20$^0$C (10 wk –20$^0$C), or extracted and analyzed after 24 hours at room temperature (24 h RT) or 48 hours at –20$^0$C (48 h -20$^0$C); or after 1 to 3 cycles of freezing at –20$^0$C and thawing at room temperature (freeze-thaw). ****** Ketoprofen, 1 mg/ml in methanol.

**Robustness:**

Robustness was evaluated by slightly altering the strength of glacial acetic acid (range of 0.18–0.22%) and acetonitrile proportion (range of 48–52%) in mobile phase. No significant effects were observed on the retention time, or shape and area of ketoprofen and the IS. Further, the chromatographic resolution and peak responses were stable over about 700 injections of processed plasma samples using a single column. Furthermore, no significant differences were observed when the assay was performed by two different operators on other apparatus from the same manufacturer.

**DISCUSSION**

We developed and validated a sensitive specific, accurate, and precise HPLC assay for the determination of therapeutic levels of ketoprofen in human plasma and applied the assay to obtain comprehensive data on the stability of ketoprofen under various laboratory conditions.

Previously reported ketoprofen assays required pH adjustment of plasma sample[5,8,10,16,21], pre column dervatazation[9,12,21], solid-phase extraction[3,6,12,22,25], chiral columns[3,6,9,10,12,15,25], or equipment that may not be readily available or need specialized training[22-25]. Further, some suffered from relatively low sensitivity (range from 0.1-1 µg/ml)[5,6,9-11,14,15,18,19], low recovery (79% and 83%)[11,14], long chromatographic run time(13, 20, 25 min)[6,7,14], or from not being validated to measure ketoprofen level in human plasma[4,7,8,10,13,17,18,20,22]. Furthermore, they provided only limited data on the stability of ketoprofen during the analytical process[3, 5-9, 11, 13-18, 20, 23].

The current assay has the following overall advantages over previously published assays: using one step liquid-liquid extraction, a short run time of 10 min, a high sensitivity of 0.01 µg/ml, and a high extraction recovery of 96%. Using the assay, we obtained extensive data on the stability of ketoprofen and the IS under various laboratory conditions. We also successfully used the assay to determine ketoprofen level in the clinically relevant range in a volunteer sample. The current assay doesn't measure ketoprofen metabolites and is not an enantioselective, however, ketoprofen metabolites are inactive and measuring enantiomers is usually not clinically relevant.

In conclusion, the data expand the available information on ketoprofen stability and indicate the superiorly of the described assay over previously reported assays, for ketoprofen therapeutic drug monitoring and bioequivalence studies.

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