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Short Communication

Simultaneous determination of flurbiprofen and its hydroxy metabolite in human plasma by liquid chromatography-tandem mass spectrometry for clinical application

Hye-In Lee[a,1], Chang-Ik Choi[b,1], Ji-Yeong Byeon[a], Jung-Eun Lee[a], So-Young Park[a], Young-Hoon Kim[a], Se-Hyung Kim[a], Yun-Jeong Lee[a,c], Choon-Gon Jang[a], Seok-Yong Lee[a,*]

[a] School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[b] College of Pharmacy, Dongguk University-Seoul, Goyang 410-820, Republic of Korea
[c] College of Pharmacy, Dankook University, Cheonan 330-714, Republic of Korea

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ABSTRACT

Flurbiprofen (FLB) is one of the phenylalkanoic acid derivatives of non-steroidal anti-inflammatory drugs used for the management of pain and inflammation in patients with arthritis. We developed and validated a rapid and sensitive high-performance liquid chromatography analytical method utilizing tandem mass spectrometry (HPLC–MS/MS) for the simultaneous determination of FLB and its major metabolite, 4′-hydroxyflurbiprofen (4′-OH-FLB), in human plasma. Probenecid was used as an internal standard (IS). After liquid–liquid extraction with methyl *t*-butyl ether, chromatographic separation of the two analytes was achieved using a reversed-phase Luna C_{18} column (2.0 mm × 50 mm, 5 μm particles) with a mobile phase of 10 mM ammonium formate buffer (pH 3.5)–methanol (15:85, v/v) and quantified by MS/MS detection in ESI negative ion mode. The flow rate of the mobile phase was 250 μl/min and the retention times of FLB, 4′-OH-FLB, and IS were 1.1, 0.8, and 0.9 min, respectively. The calibration curves were linear over a range of 0.01–10 μg/ml for FLB and 0.01–1 μg/ml for 4′-OH-FLB. The lower limit of quantifications using 100 μl of human plasma was 0.01 μg/ml for both analytes. The mean accuracy and precision for intra- and inter-run validation of FLB and 4′-OH-FLB were all within acceptable limits. The present HPLC–MS/MS method showed improved sensitivity for quantification of the FLB and its major metabolite in human plasma compared with previously described analytical methods. The validated method was successfully applied to a pharmacokinetic study in humans.

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1. Introduction

Flurbiprofen (FLB), 2-(2-fluorobiphenyl-4-yl) propanoic acid, is one of the phenylalkanoic acid derivatives of non-steroidal anti-inflammatory drugs (NSAIDs) used for the management of pain and inflammation in patients with rheumatoid arthritis and osteoarthritis. FLB is primarily metabolized via hydroxylation, mediated by the cytochrome P450 2C9 (CYP2C9) isozyme, and 45% of the drug is eliminated through this metabolic pathway [1,2].

Many previously described analytical methods used for the determination of FLB in plasma samples have used high-performance thin-layer chromatography (HPTLC) [3], high-performance liquid chromatography (HPLC) coupled with ultraviolet [4–13], fluorescence [14–19], or tandem mass spectrometric detection [20,21]. However, the relatively large volumes (500 μl or more) of plasma required for these methods [3,4,7,8,14–16], the long running times (>5 min) [5–10,13–15,17–19,21], and insufficient sensitivities (quantification limit 100 ng/ml or higher) [4,6,13,17–19,21] are obstacles that prevent successful and reliable results in pharmacokinetic studies using these methods. Furthermore, only a few reports have established analytical methods for the determination of both FLB and its major metabolite, 4′-hydroxyflurbiprofen (4′-OH-FLB), in plasma [16–18,21].

In the present study, we developed and validated a rapid and sensitive HPLC-tandem mass spectrometry (HPLC–MS/MS) method employing a simple liquid-liquid sample extraction procedure for the simultaneous determination of FLB and its metabolite in

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* Corresponding author. Tel.: +82 31 290 7718; fax: +82 31 290 7738.
E-mail address: sylee@skku.ac.kr (S.-Y. Lee).

[1] These authors contributed equally to this study.

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human plasma. This method was validated using human plasma samples by evaluating pharmacokinetic parameters, including time-dependant concentrations of FLB and 4′-OH-FLB.

2. Experimental

2.1. Chemicals and reagents

Flurbiprofen and probenecid were purchased from Sigma–Aldrich (St. Louis, MO, USA). 4′-Hydroxyflurbiprofen was purchased from Toronto Research Chemical Inc. (North York, ON, Canada). HPLC grade methanol was purchased from Burdick & Jackson (NJ, USA). HPLC grade methyl *t*-butyl ether (MTBE) was purchased from J.T. Baker (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA). Ammonium formate and hydrochloride (HCl) were purchased from Sigma–Aldrich. All other chemicals were of analytical grade and used without further purification.

2.2. Chromatographic conditions

Chromatography was performed on an Agilent 1200 series HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA). Separation was carried out on a 30 °C Luna C_{18} column (2.0 mm × 50 mm, 5 μm, Phenomenex Inc., Torrance, CA, USA). The mobile phase consisted of 10 mM ammonium formate buffer (pH 3.5) and methanol (15:85, v/v) at a flow rate of 250 μl/min. The autosampler was maintained at 4 °C and the total run time for each sample analysis was 2.5 min.

2.3. Mass spectrometry

Mass spectrometric detection was performed using an API 3200 tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, ON, Canada) equipped with an electrospray ionization (ESI) source. The mass spectrometer was operated in negative ion mode and the tandem mass spectrometry conditions for FLB, 4′-OH-FLB, and the internal standard (IS, probenecid) were optimized by carrying out full scans in negative ion detection mode. The detection and quantification of FLB, 4′-OH-FLB, and IS were performed in multiple reaction monitoring (MRM) mode. Quadrupoles Q1 and Q3 were set to unit resolution. Data acquisition and quantitation were carried out using Analyst software version 1.4.2 (Applied Biosystems/MDS SCIEX).

2.4. Preparation of standard solutions and quality control samples

Stock solutions of 1 mg/ml FLB, 4′-OH-FLB, and IS were prepared by first dissolving the compounds in deionized water. Standard working solutions (0.1, 0.5, 2.5, 10, 25, 50, and 100 μg/ml for FLB and 0.1, 0.5, 1, 2.5, 5, and 10 μg/ml for 4′-OH-FLB) were prepared by diluting stock solutions with deionized water. The working solutions were used for daily preparation of standard calibrators at concentrations of 0.01, 0.05, 0.25, 1, 2.5, 5, and 10 μg/ml for FLB and 0.01, 0.05, 0.1, 0.25, 0.5, and 1 μg/ml for 4′-OH-FLB in human plasma. The quality control (QC) working solution was prepared in the same way as the standard working solutions, and QC samples at concentrations of 0.01, 0.03, 0.6, and 9 μg/ml for FLB and 0.01, 0.03, 0.15, and 0.9 μg/ml for 4′-OH-FLB were prepared by diluting the stock and working solution with blank human plasma. The working IS solution (probenecid 20 μg/ml) was prepared by diluting the stock solution with deionized water. All stock, standard, working, and QC working solutions were stored at 4 °C.

2.5. Sample preparation

All samples were stored in a freezer at −70 °C until needed, and thereafter allowed to thaw at room temperature before processing. Briefly, 100 μl of each plasma sample, 10 μl of IS solution (probenecid, 20 μg/ml), and 100 μl of 5 M HCl were added to glass tubes. After brief vortexing, 2 ml of MTBE were added and the mixture was vortexed for 30 s. After centrifugation at 3000 rpm for 10 min, the organic layer was transferred to a new glass tube and evaporated to dryness under a gentle stream of nitrogen gas at 50 °C. The resulting residue was reconstituted with 300 μl of the mobile phase and a 5 μl aliquot was injected into the HPLC column.

2.6. Method validation

The validation was performed based on ‘Guidance for Industry: Bioanalytical Method Validation’ from the United States Food and Drug Administration (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf).

2.6.1. Selectivity and linearity

Selectivity was assessed by comparing the chromatograms of six different batches of plasma obtained from six subjects. The linearity of the method was evaluated using five different calibration curves (0.01–10 μg/ml for FLB and 0.01–1 μg/ml for 4′-OH-FLB). The calibration curves were obtained by plotting the area ratios of each analyte and IS *vs.* the concentration of analyte by least-squares linear regression with $1/x$ (where x represents the concentration of each analyte in μg/ml) as the weighting factor. The lower limit of quantification (LLOQ) was defined as the lowest concentration yielding a signal to noise ratio of at least 10 with a coefficient of variation (CV) <20% and accuracy of 80–120%.

2.6.2. Accuracy and precision

The intra-run and inter-run precisions were determined by replicate analysis of five sets of QC samples that were spiked with four different concentrations of FLB and 4′-OH-FLB within one day for intra-run or on five consecutive days for inter-run. Precision was determined as the CV and accuracy was defined as the relative standard error (RSE (%) = measured concentration/targeted concentration × 100).

2.6.3. Matrix effect and recovery

Matrix effect and recovery tests were performed in triplicate for at three different QC sample concentrations (0.03, 0.6, and 9 μg/ml for FLB and 0.03, 0.15 and 0.9 μg/ml for 4′-OH-FLB). The matrix effect was determined by extracting blank human plasma from six different sources and then reconstituting the final extract in the injection solvent, which contained known amounts of each analyte and IS. Absolute recoveries for two analytes in normal plasma were determined by extraction from blank human plasma samples spiked with the analytes. After extraction, recoveries were calculated by comparing the responses of plasma QC samples that were spiked with analytes prior to extraction with the response of those that were spiked with blank plasma.

2.6.4. Stability

The stability of FLB and 4′-OH-FLB in human plasma was evaluated in triplicate using three different concentrations of the QC sample (0.03, 0.6, and 9 μg/ml for FLB and 0.03, 0.15, and 0.9 μg/ml for 4′-OH-FLB). For short-term stability, frozen plasma samples (−70 °C) were kept at room temperature for 4 h before sample preparation. The freeze–thaw stability of each analyte was determined over three freeze-thaw cycles within three days. In each freeze–thaw cycle, the spiked plasma samples were frozen for 24 h

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