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Short Communication

Liquid chromatography-tandem mass spectrometry for the quantification of flurbiprofen in human plasma and its application in a study of bioequivalence

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ABSTRACT

A simple, quick and accurate LC-MS/MS method for the quantification of flurbiprofen in human plasma with indomethacin as internal standard (IS) was developed and validated. Samples were treated with methanol to precipitate proteins, then separated on a Ultimate C_{18} column (5 μm, 2.1 × 50 mm) with a gradient elusion process. Mobile phase A was comprised of water and formic acid, mobile phase B was comprised of acetonitrile and formic acid. Multi reaction monitoring (MRM) signals were saved on a negative ionization electrospray mass spectrometer. The calibration curve showed good linearity in the range of 40.00–10000.00 μg/L (r^2 = 0.998). Intra-day RE was 0.2–2.2%. Inter-day RE was 0.5–3.4%. The samples showed good stability under the study conditions. No significant matrix effect was observed. The established method was then applied to a bioequivalence study of a flurbiprofen axetil formulation.

1. Introduction

Flurbiprofen axetil is a targeted nonsteroidal anti-inflammatory drug, mainly used in surgery and cancer pain management. Lipid microspheres as the carrier get preferentially delivered to lesion tissues and tumor cells after injecting into the systemic circulation. Flurbiprofen axetil, is then released from the lipid microspheres, and rapidly hydrolyzed to flurbiprofen by carboxyl esterase in less than 5 min [1]. The mechanism of flurbiprofen is to inhibit the synthesis of prostaglandin. It demonstrated comparable efficacy over other NSAIDs (e.g., aspirin, indomethacin, ibuprofen, naproxen, and diclofenac) [2]. Flurbiprofen can be used as an in vivo probe for CYP2C9 activity [3].

Several HPLC methods have been reported for the determination of flurbiprofen in human plasma. They vary in sample treatment, separation condition and detector use. Albert KS et al. [4] reported a method of extraction with pentane-ether (80:20). Askholt et al. [5] reported a method of detection of UV spectrometry. Hutzler et al. [3] reported a method of fluorescence detection. With advancements in instrumentation, methods with no extraction procedure have become popular [3]. But such methods still suffer from long analytic time [6]. In the presented study we utilized LC-MS/MS, which has a better selectivity, thus the time necessary for separation was significantly reduced.

The objective of the present study was to develop and validate a rapid, selective, accurate and reliable LC-MS/MS method of flurbiprofen and apply this method to a bioequivalence study.

2. Materials and methods

2.1. Chemicals and regents

Flurbiprofen standard reference (chemical purity: 99.7%, lot No. 100725-200401), the IS (indomethacin) (chemical purity: 100.0%, lot No. 100258-200403) were purchased from National Institutes for Food and Drug Control (Beijing, China). Flurbiprofen axetil injection for test (5 mL: 50 mg, lot No. 130802) was supplied by Wuhan Docan Pharmaceutical Co., Ltd. (Wuhan, China). And the reference formulation of the flurbiprofen axetil injection (5 mL: 50 mg, lot No. 5173 K) was supplied by Beijing Tide Pharmaceutical Co., Ltd. (Beijing, China).Water was purified by a PURELAB classic (ELGA,

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England). Formic acid was MS grade (Fluka, Switzerland). Methanol and acetonitrile were HPLC grade.

2.2. Instrumentation

The LC-MS/MS system consisted of a Shimadzu UFLC LC-30 AD chromatography system (Shimadzu, Japan) and a QTRAR 4500 mass spectrometer (AB SCIEX, USA) equipped with electrospray ionization (ESI) source system. The analytes were separated on an Ultimate C18 column (5 μm, 2.1 × 50 mm) connected with a pre-column (4 × 3.0 mm I.D. Phenomenex). Mobile phase A was water-formic acid (99.9:0.1, v/v), and mobile phase B was acetonitrile-formic acid (99.9:0.1, v/v). Gradient elution was performed with 60:40 A-B for 0.1 min, 60:40 A-B to 15:85 A-B for 0.5 min, 85% B to 95% B for 0.4 min, 1 min hold at 95% B, return to 60:40 A-B for 0.1 min, 2.5 min run time, at a flow rate of 0.4 mL/min. The column temperature was set 40 °C. A 5 μL sample was injected into the sampling system with the auto-sampler conditioned at 4 °C.

The multiple reaction monitoring (MRM) transitions were performed at m/z 242.9→198.7 for flurbiprofen and m/z 355.9→312.0 for indomethacin (IS). No additional transitions were looked at. Optimized values for collision energy (CE), declustering potential (DP), entrance potential (EP) and collision exit potential (CXP) were −12 V, −26 V, −8 V and −8 V for flurbiprofen, and −20 V, −30 V, −10 V and −10 V for indomethacin (IS), respectively. Curtain gas, Ion spray voltage, source temperature, Ion source Gas1 and Gas 2 were 30 psi, −4500 V, 400 °C, 40 psi and 40 psi.

2.3. Preparation of calibration standards and quality control samples

Stock solutions were prepared by dissolving flurbiprofen and indomethacin (IS) in methanol to yield 1.0 mg/mL, 99,400 μg/L solutions, respectively. Flurbiprofen stock solution was diluted with methanol to get a series of standard working solutions, 400–100,000 μg/L. A 49,700 μg/L solution of IS was prepared by diluting the stock IS solution.

Calibration standards of flurbiprofen at concentrations of 40.0, 100, 300, 900, 2500, 5000, 10,000 μg/L were prepared by mixing 20 μL different standard working solutions, 20 μL IS solution (indomethacin 49,700 μg/L) and 200 μL blank human plasma in a 1.5 mL plastic vial. Similarly, quality control (QC) samples (low QC, med QC and high QC) were prepared by spiking blank human plasma at concentrations of 100, 1000 and 8000 μg/L.

2.4. Sample preparation

A human plasma sample (200 μL) was placed in a 1.5 mL plastic vial and then 20 μL of IS (49,700 μg/L) was added and mixed with 780 μL methanol by vortexing for 30 sec. The mixture was centrifuged at 12,000 rpm for 10 min. Finally, the supernatant liquid (5 μL) was transferred to a sample vial for later separation with LC.

2.5. Method validation

The validation of specificity, linearity, precision and accuracy, recovery and matrix effect, stability, dilution and carry-over of the presented method were carried out. Procedures were compliant with guidelines for bioanalytical method validation published by the US FDA [7] and other guidelines [8].

Selectivity of flurbiprofen over interference from endogenous substances was assessed. Selectivity of flurbiprofen over interference from flurbiprofen axetil and flurbiprofen glucuronide was assessed. 6 blank human plasma samples were analyzed under the test condition. Absence of interfering components is accepted where the response is lower than 20% LLOQ for flurbiprofen and 5% for the IS. A selected subset of samples was subject to additional analysis. These samples were treated with the same preprocessing progress. These samples were separated under the same elution process. Selected Ion Monitoring (SIM) [9] of m/z 419.1 was used to see if flurbiprofen glucuronide should exist. SIM of m/z 329.1 was used to see if flurbiprofen axetil should exist. Neither should peak at the retention time of flurbiprofen. Neutral loss of 176 was used to see if flurbiprofen glucuronide undergo source induced fragmentation to flurbiprofen. Neutral loss of 86 was used to see if flurbiprofen axetil should undergo source induced fragmentation to flurbiprofen. No related peaks should be detected at the retention time of flurbiprofen.

A calibration curves was constructed by plotting the peak area ratios of flurbiprofen to the IS against the concentrations of flurbiprofen. A replicate was conducted using the same standards after all samples were processed. And weighted least-squares linear regression with $1/x^2$ as weighting factor was adopted to derive the overall equation from data points gathered both pre- and post-sample.

Accuracy and precision were assessed in five samples at three different QC levels on the same day and among three different days. RE should be within ±15% and the intra- and inter-day RSD should not exceed 15%.

The extraction recovery was evaluated at three QC levels and reckoned by comparing the peak areas received from plasma samples with the analytes spiked before extraction to those spiked after extraction. Matrix effect was determined by peak areas of flurbiprofen in two QC levels (100, 8000 μg/L), calculated as a percentage of the response of samples prepared by spiking flurbiprofen and the IS with drug-free human plasma (blank matrix) extracts from six different sources to those spiked with mobile phase.

Stability was investigated at three QC levels under 4 different conditions: 24 h in the autosampler, 24 h at room temperature, three cycles of freeze thaw, and 32 days at −80 °C, with 5 replicates under each condition.

Carry-over was assessed by injecting a blank plasma sample following a sample of the LLOQ and a sample of the ULOQ. This measurement was repeated 3 times. All carry-over peaks should not exceed 15% LLOQ.

The dilution integrity was demonstrated by diluting plasma samples with flurbiprofen 12,500 μg/L 2.5- fold by blank plasma, five replicates. RE and RSD should be within ±15% and below 15% respectively.

2.6. Bioequivalence study

The proposed method was applied to a Bioequivalence study of flurbiprofen axetil injection among healthy male Chinese volunteers. The study protocol was approved by the Ethics Committee of Huazhong University of Science & Technology and a written informed consent was obtained from all volunteers.

Subjects ($n = 24$) were randomly assigned (1:1) into two groups, and received a single dose of either test or reference formulation of a 5 mL (i.v.in not longer than 2 min) flurbiprofen axetil, and a single dose of the alternate formulation after a 7-day washout period. Subjects were fasted for 12 h prior to administration. Blood samples were collected in heparinized tubes immediately before (0 h) and 0.17, 0.25, 0.33, 0.5, 1, 1.5, 2, 4, 6, 8, 12, and 24 h after administration. Samples were centrifuged at 3000 rpm for 10 min and plasma was separated and stored at −70 °C until analysis.

A non-compartmental method was used to evaluate the pharmacokinetic parameters. Following guidelines [10] bioequivalence was assessed by means of an analysis of variance (ANOVA) and calculating the standard 90% confidence intervals (90% CIs) of the ratio