Liquid chromatography–mass spectrometric method for the sensitive determination of niflumic acid in human plasma and its application to pharmacokinetic study of talniflumate tablet

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ABSTRACT

A sensitive LC–MS method was developed and validated for the determination of niflumic acid (NFA), the active metabolite of the talniflumate formulation, in human plasma. The analyses were performed on C_{18} column using acetonitrile–ammonium acetate buffer (pH 5.7, 40:60) as a mobile phase with quadrupole MS detection of NFA at m/z 281 in a negative ion-monitoring mode. Calibration curve was linear in the concentration range of 1–1000 ng/mL in human plasma. The higher sensitivity of LC–MS allowed low concentrations of NFA to be determined at initial drug absorption and terminal elimination phases following oral administration of talniflumate tablet.

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1. Introduction

Talniflumate [3-phthalidyl ester of 2-([3-(trifluoromethyl) phenyl]amino)-3-pyridinecarboxylic acid] is a prodrug of niflumic acid [2-(α,α,α-trifluoro-m-toluidino) nicotinic acid] (Fig. 1), which is a potent analgesic and anti-inflammatory drug widely used to treat rheumatoid diseases [1]. Talniflumate was synthesized by esterifying the carboxyl group of niflumic acid (NFA) with a phthalidyl moiety to lower the ulcerogenic effect, and also has a greater anti-inflammatory effect than NFA [2]. Furthermore, recently, talniflumate has also attracted attention as a mucoregulator for the treatment of cystic fibrosis, chronic obstructive pulmonary disease, and asthma [3].

Because talniflumate exerts its activity in the body by being converted to NFA by esterase, NFA is a target molecule for bioavailability and pharmacokinetic studies of talniflumate formulations. Several methods of determining NFA levels in biological fluids have been published, e.g., luminescence spectroscopy [4], gas chromatography [5], and high-performance liquid chromatography (HPLC) [6–11]. Among them, HPLC methods with UV absorbance detection have been used for the determination of NFA in human plasma for the pharmacokinetic and bioequivalence studies of talniflumate tablets [8–11]. These methods showed a lower limit of quantitation (LLOQ) of 10–20 ng/mL for the NFA. However, this sensitivity was found to be insufficient for the determination of NFA following oral administration of talniflumate tablet.

The purpose of this study was to develop LC–MS method as a sensitive analytical method enough to determine NFA levels in biological fluids. The developed method was validated for the determination of NFA in human plasma and applied to a pharmacokinetic study in human volunteers following the oral administration of a talniflumate tablet.

2. Experimental

2.1. Materials and reagents

NFA and indomethacin were purchased from Sigma (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were obtained from J.T. Baker (Philipsburg, NJ, USA). Somalgen tablets (talniflumate 370 mg) were obtained from Kun-Wha Pharm. Co. Ltd. (Seoul, Korea). All other chemicals were of analytical grade.

2.2. Preparation of standard samples

A stock solution of NFA was prepared in methanol at a concentration of 1 mg/mL and the working standard solutions were prepared by diluting an aliquot of stock solution with methanol. The working solution of indomethacin, internal standard (IS), at 10 μg/mL was prepared by diluting the stock solution (1 mg/mL
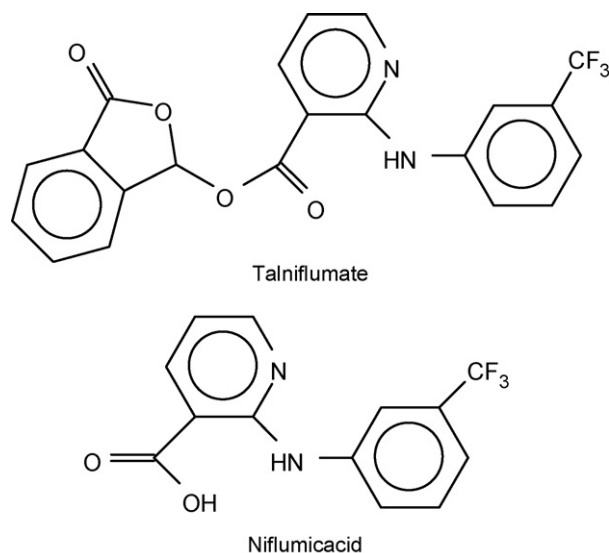
Fig. 1. Chemical structures of talniflumate and niflumic acid (NFA).

indomethacin in methanol) with methanol. As the indomethacin has been successfully used in the previous studies [9–11], it was selected as IS in this study. Calibration standards of NFA (1, 5, 10, 50, 100, 200, 500 and 1000 ng/mL) were prepared by spiking the appropriate amounts of working standard solutions into eight lots of drug-free human plasma. The quality control (QC) samples at 1, 20, 150 and 800 ng/mL for NFA were prepared by adding 10 μL of the appropriate working standard solutions of NFA (0.05, 1.0, 7.5 and 40 μg/mL) and 50 μL of the working solution of IS to 490 μL of drug-free human plasma. All solutions were stored in a freezer at −20 °C until required for analysis.

2.3. Preparation of plasma samples

A volume of 50 μL of IS (10 μg/mL) was spiked into 0.5 mL of human plasma. To remove plasma proteins, 0.9 mL of methanol was added to the plasma mixture. After vortex mixing for 60 s and centrifuging at 5000 rpm for 30 min at 4 °C, 10 μL of the supernatant was injected directly into the LC–MS system.

2.4. Instrumentation and LC–MS conditions

LC–MS analysis was performed using an Agilent Technologies Series 1100 LC/MSD VL system (Agilent Technologies, Palo Alto, CA, USA) with a Luna C_{18} column (150 mm × 2.0 mm i.d., 3 μm particle size; Phenomenex, Torrance, CA, USA). The mobile phase was acetonitrile–ammonium acetate (pH 5.7 adjusted with acetic acid; 10 mM) (40:60, v/v) at a flow rate of 0.2 mL/min. The column temperature was maintained at 25 °C. The mass spectrometer was operated in negative ion mode using the following conditions: drying gas (N_2) flow 10 L/min, drying gas temperature 350 °C, nebulizer pressure 276 kPa, and capillary voltage 3.5 kV. The fragmentor voltage used was 100 V. LC–MS was performed in selected ion monitoring (SIM) mode selecting the deprotonated molecular ions $[M-H]^-$ m/z 281.0 for NFA and $[M-H]^-$ m/z 356.0 for IS (indomethacin), respectively.

2.5. Method validation

The method was validated according to the Guidance for Industry, Bioanalytical Method Validation reported by U.S. FDA [12]. Specificity was assessed by extracting samples of six different

batches of blank human plasma, a zero sample (spiked with IS only), and then comparing the results of plasma samples spiked with IS and NFA (1 or 200 ng/mL) in calibration standard. Ion chromatograms were visually inspected for interfering chromatographic peaks from endogenous substances.

NFA calibration standards at concentrations of 1, 5, 10, 50, 100, 200, 500 and 1000 ng/mL were assayed. The linearity of the calibration curve was confirmed by plotting peak-area ratios of NFA to IS versus NFA concentrations with least-squares linear regression analysis. Intra- and inter-day precisions and accuracies of the LC–MS method were determined by analyzing QC samples at concentrations of 1, 20, 150 and 800 ng/mL for NFA. Intra-day precision was determined by repeating the analysis of each QC sample five times in a single day, and inter-day precision and accuracy were determined by repeating this analysis on five consecutive days. The concentration of each sample was determined by using calibration standards prepared on the same day. Limit of detection (LOD) and lower limit of quantitation (LLOQ) were defined as the minimum concentrations corresponding to 3 and 10 times the signal-to-noise ratio (S/N), respectively.

The absolute matrix effect was assessed by comparing mean peak areas of analyte at three QC concentrations (20, 150 and 800 ng/mL) spiked into deproteinized extracts from five different lots of blank human plasma samples (set 2) to mean peak areas for neat solutions of the analytes in methanol (set 1) [13]. The variabilities in the peak areas for analytes spiked after protein precipitation into five different plasma extracts (set 2), expressed as CVs (%), were considered as measures of the relative matrix effect. Recovery of NFA was determined by comparing mean peak areas of analyte spiked before extraction into the same five different sources as set 2 (set 3) with those of the analyte spiked post-extraction into different blank plasma lots at three QC concentrations (set 2).

To test the short- and long-term stabilities of extracted niflumic acid, three QC samples (20, 150 and 800 ng/mL) were analyzed after several freeze–thaw cycles by thawing at room temperature for 4 h and freezing for 24 h. The short-term stability was determined during the storage for 12 h at room temperature, 4 and −20 °C. Long-term storage stability at −70 °C was determined after 20 days.

2.6. Pharmacokinetic study of talniflumate

The pharmacokinetic study was performed by the approval of the Korean Food and Drug Administration (KFDA) and the Institutional Review Board (IRB) on human studies at Wallace Memorial Baptist Hospital (Busan, Korea). Healthy male Korean volunteers aged 19–28 years and weighing from 60 to 85 kg were selected for this study after passing a clinical screening procedure including hematology, biochemistry, electrolytes and urine analysis. After an overnight fast more than 12 h, the subjects were given a single dose of one talniflumate tablet (370 mg) with 240 mL of water. At 4 h after the oral administration, all subjects were given standardized meals. The subjects were not allowed to remain in the supine position or to sleep until 8 h after the oral administration. Approximately 5 mL of blood samples were collected via the cannula at the following times; pre-dose, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 9, 12 and 15 h after the administration. The plasma samples were separated by centrifugation at 1000 × g for 10 min and stored at −70 °C until the LC–MS analysis.

The pharmacokinetic parameters including maximum plasma concentration (C_{max}), time point of maximum plasma concentration (T_{max}), area under the plasma concentration versus time curve from 0 h to the last measurable concentration (AUC_{0-t}), area under the plasma concentration versus time curve from 0 h to infinity (AUC_{∞}) and half-life of drug elimination during the terminal phase