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Quantitation of niflumic acid in human plasma by high-performance liquid chromatography with ultraviolet absorbance detection and its application to a bioequivalence study of talniflumate tablets

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Abstract

A rapid and simple HPLC method with UV detection (288 nm) was developed and validated for quantitation of niflumic acid in human plasma, the active metabolite of talniflumate. After precipitation with 100% methanol containing the internal standard, indomethacin, the analysis of the niflumic acid level in the plasma samples was carried out using a reverse phase C_{18} CAPCELL PAK (5 μm, 4.6 mm × 250 mm) column. The chromatographic separation was accomplished with an isocratic mobile phase consisting of a mixture of 0.1 M sodium acetate in water and acetonitrile (37:63, v/v), adjusted to pH 6.4. This HPLC method was validated by examining its precision and accuracy for inter- and intra-day runs in a linear concentration range of 0.02–5.00 μg/mL. Stability of niflumic acid in plasma was excellent, with no evidence of degradation during sample processing (autosampler) and 30 days storage in a freezer. This validated method was successfully applied to the bioequivalence study of talniflunate in healthy volunteers.

Keywords: Talniflumate; Niflumic acid; Validation; High-performance liquid chromatography (HPLC); Bioequivalence test

1. Introduction

Niflumic acid [2-(α,α,α-(trifluoro-*m*-toluidino) nicotinic acid] is a potent analgesic and anti-inflammatory drug, which is widely prescribed for the treatment of rheumatoid diseases [1]. It shows rapid absorption followed by extensive metabolization, essentially involving hydroxylation or glucuro-conjugation [1]. Talniflumate [2-{[3-(trifluoromethyl)phenyl]amino}-3-pyridinecarboxylic acid 1,3-dihydro-3-oxo-1-iso-benzofuranyl ester] was designed as a prodrug of niflumic acid with potential application in a clinical context. Considering that talniflumate is less irritant to gastrointestinal mucosa than niflumic acid, talniflumate seems to be advantageous over niflumic acid, in terms of its activity and side effects [2].

Several methods have been described for the determination of niflumic acid in biological fluids, including gas chromatography [3] and high-performance liquid chromatography (HPLC) with ultraviolet [4–8]. However, neither of these methods could be used in this case, due to their low sensitivity and the low efficiency of the sample pretreatment process. Therefore, a more sensitive and simpler HPLC method is needed for the determination of niflumic acid in clinical tests.

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The bioequivalence of two formulations of the same drug implies their equivalence with respect to the rate and extent of absorption. While the area under the plasma concentration–time curve from 0 h to the last measurable concentration (AUC_{0-t}) generally serves as the characteristic of the extent of absorption, the peak concentration (C_{max}) and the time of its occurrence (T_{max}), reflect the rate of absorption, especially in the case of fast-releasing drug formulations [9–10]. Following the introduction of a new formulation of talniflumate by the Daewon Pharmaceutical Co. Ltd. (Seoul, Korea), in this study the bioequivalence of the two formulations, PALIMA® tablets (test medication) and SOMALGEN® tablets [reference medication, manufactured by Kun Wha Pharmaceutical Co. Ltd. (Seoul, Korea)] was assessed in 24 healthy male Korean volunteers.

The purpose of this study was two-fold. Firstly, to develop a more sensitive and reproducible HPLC method of analyzing niflumic acid in human plasma, having a lower limit of quantitation (LLOQ) of 0.02 μg/mL and employing a simpler, one-step pretreatment consisting of methanol precipitation. Secondly, to use this method to determine the pharmacokinetic parameters of the two brands of talniflumate 370 mg and, then, to evaluate the bioequivalence between the two brands by comparing these parameters statistically. PALIMA® was used as the test product, while SOMALGEN® was used as the reference product.

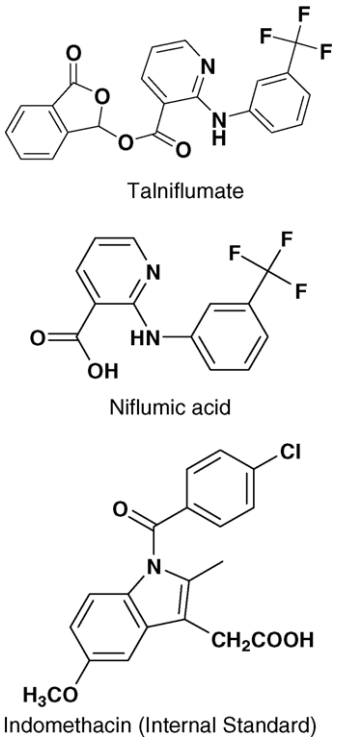

Fig. 1. Chemical structures of talniflumate, niflumic acid and internal standard (indomethacin).

2. Experimental procedure

2.1. Drugs and reagents

Niflumic acid and indomethacin (Fig. 1) were purchased from Sigma–Aldrich Korea. The solvents, i.e., methanol, acetonitrile and acetic acid (all HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). A Milli-Q® (Millipore Co., Milfora, MA, USA) water purification system was used to obtain the purified water used for the HPLC analysis. All other chemicals and solvent were of the highest analytical grade available. The test medication, PALIMA® [370 mg talniflumate tablet, Daewon Pharm. Co. Ltd. (Seoul, Korea)] and the reference medication, SOMALGEN® [370 mg talniflumate tablet, Kun Wha Pharm. Co. Ltd. (Seoul, Korea)] were supplied in the form of tablets.

2.2. Calibration standards and quality control (QC) samples

The stock solution (1 mg/mL) of niflumic acid was prepared in methanol. The niflumic acid stock solution was dissolved in drug-free heparinized plasma to obtain a concentration of 0.02, 0.05, 0.20, 0.50, 1.00, 2.00 and 5.00 μg/mL. The quality control samples were prepared in pool, at concentrations of 0.02 μg/mL (low), 0.2 μg/mL (medium), 2 μg/mL (high), as a single at each concentration, and then divided in aliquots that were stored in the freezer at −70 °C until analysis. The internal standard (IS) stock solution was diluted with methanol to a final concentration of 10 μg/mL. These standard solutions were employed for the preparation of the calibration graphs.

2.3. Preparation of plasma samples

After thawing at room temperature, an aliquot of each sample (500 μL) was pipetted into an Eppendorf tube, and indomethacin (IS) solution (50 μL, 10 μg/mL) was added. After vortexing briefly, 900 μL of cold methanol was added to each sample and the samples were vortexed again for 60 s and centrifuged for 20 min at 3000 rpm. After centrifuging, 100 μL of the sample was injected into the HPLC system equipped with a UV detector and the peak height was recorded.

2.4. Chromatographic conditions

The HPLC system consisted of a Waters model 515 HPLC pump, a model 717 WISP autoinjector and a model 486 tunable absorbance detector set to 288 nm (Waters Assoc., Milford, MA, USA). The chromatographic data was collected and analyzed using Millenium Chromatography Manager (Waters, version 2.15). The chromatography for the separation and determination of the drug was carried out by applying the samples to a Shiseido C_{18} CAPCELL PAK (5 μm, 4.6 mm × 250 mm) column at 30 °C. The mobile phase was prepared by mixing acetonitrile with 0.1 M sodium acetate (pH 6.4 with acetic acid) at a ratio of 37:63 (v/v), and

a flow rate of 1.0 mL/min was found to be adequate for the analysis of the samples. A constant slow bubbling of helium through the mobile phase during operation was required to keep the level of oxygen in the system at a minimum.

2.5. Validation

2.5.1. Linearity (calibration curves)

A calibration curve was constructed from a blank sample (a plasma sample processed without IS), a zero sample (a plasma processed with IS) and seven non-zero samples covering the total range (0.02–5.00 μg/mL), including lower limit of quantitation. Such calibration curves were generated on seven consecutive days. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the normal value except LLOQ, which was set at 20%.

2.5.2. Specificity

Six randomly selected blank human plasma samples, which were collected under controlled conditions, were carried through the similar precipitation procedure and chromatographed to determine the extent to which endogenous plasma components may contribute to interference with the analyte or the internal standard.

2.5.3. Recovery

Recovery of niflumic acid in plasma was evaluated by comparing the mean detector response of different QC samples extracted with those prepared by adding compound to post-extracted drug free plasma at the corresponding concentrations. Similarly, the recovery of IS from plasma was also evaluated.

2.5.4. Accuracy and precision

In order to assess the intra-day coefficient of variation (CV) and accuracy of the plasma samples, the samples of niflumic acid were spiked into human plasma at final concentrations of 0.02, 0.05, 0.20, 0.50, 1.00, 2.00 and 5.00 μg/mL and indomethacin solution (50 μL, 10 μg/mL). The precision and accuracy of the inter-day assay were assessed at the same concentration and calculated for five different days. The limit of detection (LOD) and lower limit of quantitation (LLOQ) were defined as the minimum concentration at which the signal-to-noise ratio (S/N) = 3 and 10, respectively. The precision and accuracy in 0.02 μg/mL were within 20%, as required by the KFDA [11].

2.5.5. Stability

To test the short- and long-term stability of extracted niflumic acid, three QC samples, containing low (0.02 μg/mL), medium (0.20 μg/mL) and high (2.00 μg/mL) concentrations, were determined after several freeze and thaw cycles, by thawing at room temperature for 2–3 h, frozen for 12–24 h. The short-term stability of the extracted samples during storage for 24 h at 4 °C, room temperature and −20 °C were also determined. Moreover, the long-term storage stability at −70 °C was determined after 30 days.

2.6. Bioequivalence test of talniflumate

The study population consisted of 24 healthy male Korean volunteers with an average age of 23.04 years and an average weight of 67.88 kg. The volunteers were selected after passing a clinical screening procedure including a physical examination and laboratory tests (blood analysis; hemoglobin, hematocrit, WBC, platelet, differential counting of WBC, blood urea nitrogen, total bilirubin, cholesterol, total protein, albumin, alkaline phosphatase, glucose fasting, sGOT, sGPT, urine analysis; specific gravity, color, pH, sugar, albumin, bilirubin, RBC, WBC, and cast). The volunteers were excluded if there was any possibility of their being sensitive to this type of medication, had a history of any illness of the hepatic, renal or cardiovascular systems, or a history of excessive alcohol intake or other medications. This was done to ensure that the existing degree of variation would not be due to an influence of illness or other medications. All of the volunteers avoided using other drugs for at least 1 week prior to the study and until after its completion. They also refrained from consuming alcoholic beverages, and xanthine-containing foods and beverages for 48 h prior to each dosing and until the collection of the last blood sample. Each volunteer received an oral dose of 740 mg (two tablets) of talniflumate in a standard 2 × 2 cross-over model in a randomized order. There was a 1-week washout period between the doses. All of the participants signed a written consent form after they had been informed of the nature and details of the study in accordance with the Korean Guidelines for Bioequivalence Test [11]. The subjects were hospitalized (Kyung Hee Medical Center, Seoul, Korea) at 10.00 p.m. on the eve of the study and fasted overnight (10 h) and 4 h after each drug administration. At 7.00 a.m., their median cubital vein was cannulated and 7 mL blood samples were drawn into heparinized tubes. The doses were taken at 8.00 a.m. of each dosing day along with 240 mL of tap water. At 4 h after the oral administration, all of the 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 7 mL blood samples were collected via the cannula at the following times; predose, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 9, 12 and 15 h after the administration. On each occasion, the blood sample was centrifuged immediately, and the plasma sample was frozen at −70 °C until the HPLC analysis.

2.7. Pharmacokinetic data and statistical analysis

The pharmacokinetic parameters: maximum plasma concentration (C_{max}), time point of maximum plasma concentration (T_{max}), area under the plasma concentration–time curve from 0 h to the last measurable concentration (AUC_{0-t}), area

under the plasma concentration–time curve from 0 h to infinity (AUC_∞), elimination rate (k_e) and half-life of drug elimination during the terminal phase ($t_{1/2}$) were determined.

Based on the statistical results of the 90.0% confidence intervals for the ratios of the means of the log-transformed pharmacokinetic parameters, C_{max}, and AUC_{0-t}, conclusions were drawn as to whether the test product was bioequivalent to the reference product. Bioequivalence was to be concluded if the 90.0% confidence intervals for C_{max} and AUC_{0-t} fell within the bioequivalence range of 0.80–1.25% [11]. The descriptive statistics for the pharmacokinetic parameters were computed using WinNonlin Professional Software (version 3.0.1). For the purpose of the bioequivalence analysis, a two-way Analysis of Variance (ANOVA) was performed with the K-BE Test 2002 program [12] at a significant level of 0.05. The test and reference treatments of each study were compared with respect to the relevant pharmacokinetic variables, using an analysis of variance with the subject, treatment and period effects based on the raw data.

3. Results and discussion

3.1. Separation and specificity

Fig. 2 shows the typical HPLC chromatograms of the sample analysis. No interference with the constituents from the drug-free human plasma was observed. Niflumic acid and the internal standard were well separated from the biological background under the chromatographic conditions described above at the retention times of 7.0 and 5.8 min, respectively. The total analysis time for each run was 10 min. There were no interfering peaks in six different randomly selected samples of drug human plasma used for analysis at the retention times of either analyte or internal standard. The mobile phase that was used guaranteed good repeatability of the retention times.

3.2. Calibration and validation

The calibration curve was obtained by analyzing eight samples for a total of nine times each. The curve was linear over the whole range tested (0.02–5.00 μg/mL) and described by the following equation: $y = 3.1450x + 0.0001$ ($r^2 = 0.9998$). The intra-day accuracy of the method of determining niflumic acid ranged from 80.57% to 103.61%, while the intra-day precision ranged from 0.83% to 16.04%. The inter-day accuracy of the method of determining niflumic acid ranged from 97.31% to 108.84% while the inter-day precision ranged from 1.55% to 9.60% (Table 1). The lower limit of quantitation (LLOQ) of niflumic acid was 0.02 μg/mL (signal-to-noise ratio of 10) and the minimum detectable level (LOD) was 0.01 μg/mL (signal-to-noise ratio of 3). The LLOQ had a much lower value than those obtained by Avegerinos and Malamataris [7] and Kim et al. [5]. This was the lowest concentration of analyte that could be measured with both a coefficient of variation and accuracy of <20%. Moreover, this LLOQ is sufficient for pharmacokinetic studies. The precipitation recovery values obtained for niflumic acid were shown to be consistent and reproducible.

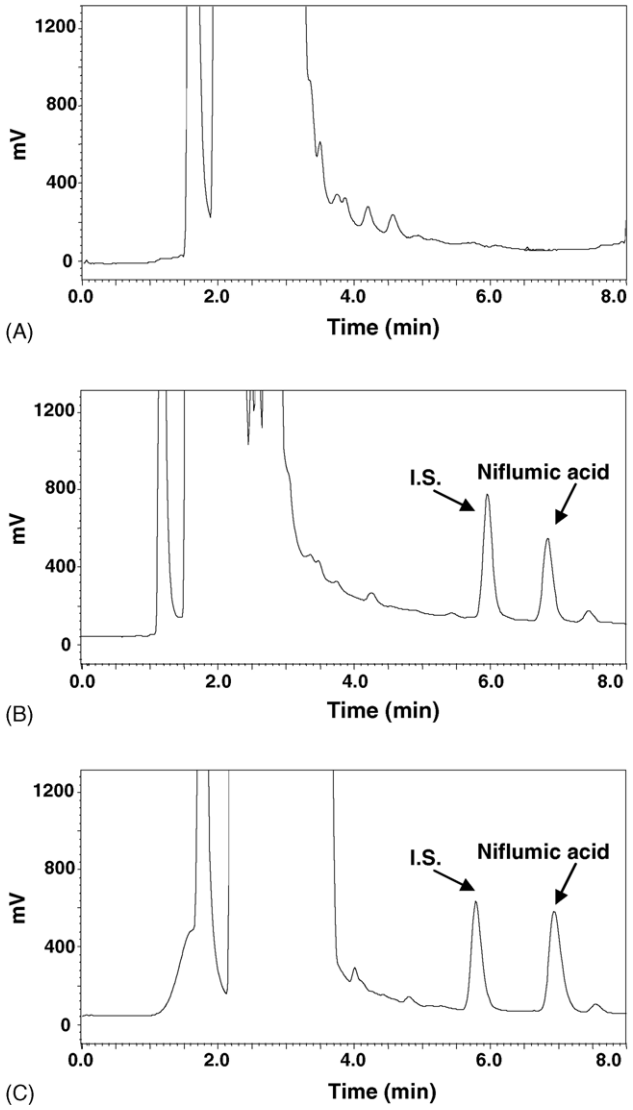

Fig. 2. Chromatogram of (A) blank human plasma, (B) plasma spiked with 0.2 μg/mL niflumic acid and indomethacin (50 μL, 10 μg/mL), and (C) plasma from a volunteer 4 h after the oral administration of SOMALGEN® (two tablets).

Table 1
Precision and accuracy for the determination of niflumic acid in human plasma

Concentration (μg/mL)	Precision (%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.02	16.04	6.69	80.57	108.54
0.05	1.69	1.55	93.83	108.84
0.20	2.00	2.33	103.61	104.84
0.50	12.31	3.57	97.74	102.87
1.00	0.83	2.53	100.88	100.51
2.00	1.09	2.42	100.26	97.31
5.00	9.93	9.60	99.94	100.37

Table 2
Stability data for niflumic acid ($n = 3$ per test and each concentration)

	Theoretical concentration (μg/mL)		
	0.02	0.20	2.00
Long term			
30 days, −70 °C (%)	89.35 ± 4.32	97.82 ± 2.99	98.76 ± 2.84
Short term			
24 h, 4 °C (%)	87.38 ± 5.58	95.26 ± 3.10	95.84 ± 3.38
24 h, room temperature (%)	89.36 ± 6.62	98.87 ± 4.55	92.38 ± 2.89
24 h, −20 °C (%)	98.76 ± 4.96	101.21 ± 2.69	104.46 ± 3.55
Freeze–thaw stability (%)	88.85 ± 4.51	91.65 ± 5.56	107.54 ± 3.35

The mean recoveries of the 0.02, 0.20, and 2.00 μg/mL levels were 101.37%, 98.97% and 97.44%, respectively. The recovery of internal standard, indomethacin was 97.42% at the concentration used in the assay (1 μg/mL).

3.3. Stability

To evaluate niflumic acid stability in human plasma, drug-free plasma samples were spiked at 0.02, 0.20 and 2.00 μg/mL. After precipitation, samples were arranged in the autosampler and were analyzed. In the short-term stability study, niflumic acid was found to be stable for 24 h at 4 °C, room temperature and −20 °C. In the long-term stability study, the plasma samples spiked with niflumic acid also showed no loss of analytes when they were stored for 30 days at −70 °C. The final stability test was demonstrated after three freeze–thaw cycles. No significant deterioration of the analytes was observed under any of these conditions (Table 2).

3.4. Bioequivalence test of talniflumate products

The HPLC method described herein was applied to a bioequivalence study of two talniflumate tablet formulations. The mean (±S.D.) plasma concentration–time profiles of niflumic acid after administration of a single oral dose of 740 mg for both formulations in tablet form are shown in Fig. 3. No significant sequence effect was found for any of the bioavailability parameters, thus indicating that the cross-over design was properly performed. The geometric means of the parameters are given for the test and reference formulations, both separately for each period and as combined estimates (Table 3). The parametric point estimates for the mean of the test medication/mean of the reference medication for AUC_{0-t} and C_{max} were 0.933% and 1.006%, respectively, and the parametric 90% confidence intervals for AUC_{0-t} and C_{max} were $0.8395 < \delta < 1.0390$ and $0.9450 < \delta < 1.0727$, respectively, which were within the accepted range of 0.80–1.25% by the Korean and US Food and Drug Administration [11,13]. The detailed bioequivalence analysis for the T_{max} value under the assumption of a non-parametric model is also given. The pharmacokinetic parameters, such as AUC_{0-t}, AUC_{∞}, k_e, $t_{1/2}$, C_{max} and T_{max} of the test drug were similar to those of the reference drug, as shown in Table 3, which proves that there was no significant difference between the bioavailabilities of SOMALGEN® (reference drug) and PALIMA® (test drug).

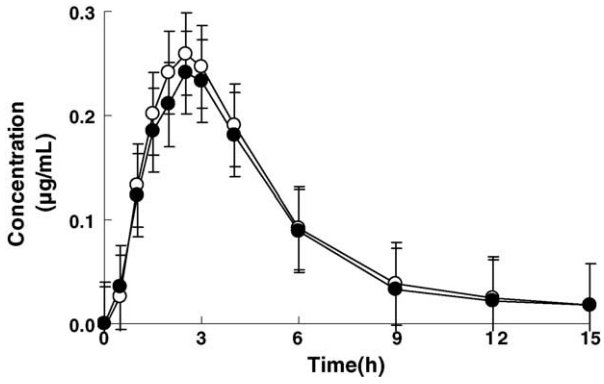

Fig. 3. Mean plasma concentration–time curves of niflumic acid following the oral administration of reference (○) and test (●) formulations at a dose of 740 mg of talniflumate to healthy, adult, male and human subjects (mean ± S.D., $n = 24$).

Table 3
Bioavailability parameters for each volunteer obtained after oral administration of SOMALGEN® and PALIMA® tablets at the talniflumate dose of 740 mg

Pharmacokinetic parameters	SOMALGEN® (mean ± S.D.)	PALIMA® (mean ± S.D.)	Confidence limit 90.0%
T_{max} (h)	2.46 ± 0.79	2.73 ± 0.87	–
C_{max} (μg/mL)	0.29 ± 0.19	0.28 ± 0.15	94.50–107.27
AUC_{0-t} (μg h/mL)	1.27 ± 0.85	1.14 ± 0.71	83.95–103.90
AUC_{∞} (μg h/mL)	1.20 ± 0.77	1.39 ± 0.87	81.37–104.78
$t_{1/2}$ (h)	0.22 ± 0.04	0.19 ± 0.06	–
k_e (h^{-1})	4.10 ± 1.49	4.73 ± 2.19	–

The 90.0% confidence intervals for the ratios of test drug to reference drug in terms of AUC_{0-t}, AUC_{∞}, and C_{max} were within the range 80.0–125.0%

4. Conclusion

In conclusion, a rapid and convenient method was developed for the determination of the active metabolite of talniflumate, niflumic acid, in human plasma. The bioequivalence of two different 370 mg talniflumate tablet formulations, based on the oral administration of a 740 mg dose consisting of two tablets, in 24 healthy, normal male volunteers was examined by monitoring its active metabolite. The statistical analysis results based on comparisons of the three pivotal parameters (AUC_{0-t}, AUC_{∞} and C_{max}) point to the bioequivalence of these two tablet formulations of talniflumate, leading to the conclusion that they may be prescribed interchangeably.

Acknowledgements

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