Simultaneous Characterization of Sofalcone and Its Metabolite in Human Plasma by Liquid Chromatography – Tandem Mass Spectrometry

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A sensitive and selective method for quantitation of sofalcone and its active metabolite in human plasma has been established using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS). Plasma samples were transferred into 96-well plate using an automated sample handling system and spiked with 10 μL of 2 μg/mL d_3-sofalcone and d_3-sofalcone metabolite solutions (internal standard), respectively. After adding 0.5 mL of acetonitrile to the 96-well plate, the plasma samples were then vortexed for 30 sec. After centrifugation, the supernatant was transferred into another 96-well plate and completely evaporated at 40 °C under a stream of nitrogen. Dry residues were reconstituted with mobile phase and were injected into a C_{18} reversed-phase column. The limit of quantitation of sofalcone and its metabolite was 2 ng/mL, using a sample volume of 0.2 mL for analysis. The reproducibility of the method was evaluated by analyzing 10 replicates over the concentration range of 2 ng/mL to 1000 ng/mL. The validation experiments of the method have shown that the assay has good precision and accuracy. Sofalcone and its metabolite produced a protonated precursor ion ($[M+H]^+$) of *m/z* 451 and 453, and a corresponding product ion of *m/z* 315 and 317, respectively. Internal standard (d_3-sofalcone and d_3-sofalcone metabolite) produced a protonated precursor ion ($[M+H]^+$) of *m/z* 454 and 456 and a corresponding product ion of *m/z* 315 and 317, respectively. The method has been successfully applied to a pharmacokinetic study of sofalcone and its active metabolite in human plasma.

Key Words : Tandem mass spectrometry, Liquid chromatography, Protein precipitation, Sofalcone, Pharmacokinetic study

Introduction

Sofalcone, 2'-carboxymethoxy-4,4'-bis(3-methyl-2-butenyloxy)chalcone, is an anti-ulcer drug. It enhances the protective qualities of mucus component of gastric mucosal barrier and inhibits growth of Helicobacter pylori.[1-3]

Recently, we have reported the measurement of sofalcone in human plasma by high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) in our previous work.[4] But, that was only for analysis of a single analyte (sofalcone) using isocratic mobile phase conditions. Since it is important to monitor metabolites as well as their parent compounds in pharmacokinetic studies, we have developed a novel analytical method for the simultaneous characterization of sofalcone and its active metabolite in human plasma prepared by protein precipitation (PP) using LC-MS/MS with a gradient. To our best knowledge, no LC-MS/MS assays have been previously reported for simultaneous analysis of sofalcone and its active metabolite.

Outstanding precision and accuracy was obtained over a wide dynamic range using this new analytical method. Additionally, its stability, recovery, matrix effects and reproducibility were also evaluated throughout our method development process. This LC-MS/MS assay is simple and offers great sensitivity and selectivity. This assay was used in our pharmacokinetic study of sofalcone and its active metabolite in human plasma that we report here in this paper.

Experimental Section

Reagents and solutions. Sofalcone and its metabolite were obtained from SK pharma Co., Ltd. (Seoul, South Korea). And internal standards were obtained from ChungAng University (Seoul, South Korea). HPLC grade methanol, acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA), and ammonium acetate and acetic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Figure 1 shows the structures of sofalcone and its active metabolite. To prepare standard stock solutions, an appropriate amount of sofalcone, sofalcone metabolite, and internal standards (d_3-sofalcone, d_3-sofalcone metabolite) were weighed accurately and dissolved in 50 mL of methanol, respectively. From these stock solutions, working standard solutions that contain 200 ng/mL to 100 μg/mL sofalcone or its metabolite were

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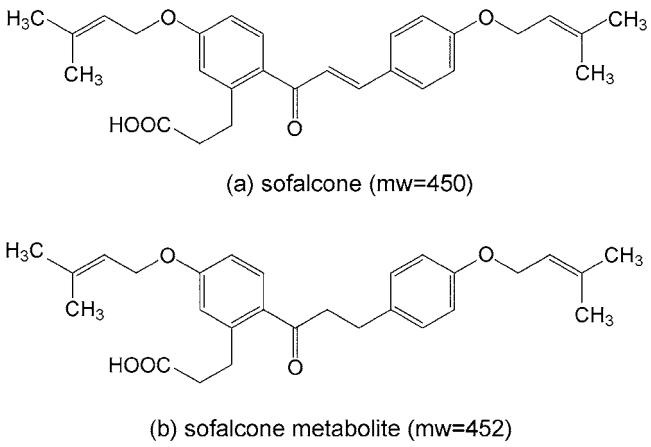

Figure 1. Structures of (a) sofalcone and (b) sofalcone metabolite.

prepared by serial dilutions with mobile phase consisted of acetonitrile/2 mM ammonium acetate, pH 6.15 (80 : 20; v/v), respectively.

Instrumentation and LC-MS/MS conditions. MS/MS was performed with a Quattro micro triple quadruple mass spectrometer (Micromass Co., Manchester, UK) equipped with an electrospray ionization (ESI) source. Samples (20 μL) were injected into the ESI source through a HPLC equipped with a autosampler (Waters HT 2795, Waters Co., Milford, USA) connected with a C_{18} Capcell Pak AQ column (2.0 × 150 mm, 5.0 μm particle) maintained at 50 °C. The mobile phase consisted of 100% acetonitrile (A) and 2 mM ammonium acetate in water (pH 5.5 with acetic acid) (B). A gradient program was used for chromatographic separation with a flow rate of 0.2 mL/min. The initial composition of mobile phase A was 40%, then increased to 55% in 13.0 min, then increased to 80% in 13.5 min and maintained for 2.5 min, followed by re-equilibration to the initial condition for 3.0 min. The total run time was 29.0 min. The electrospray ion source was maintained at 300 °C. Nitrogen nebulization was performed with a nitrogen flow of 1024 L/hr. Argon was used as collision gas at a pressure of $3.91e^{-3}$ mbar. Sofalcone and its metabolite were monitored by multiple reaction monitoring (MRM) mode with positive ion detection, the parameter settings used were: capillary voltage at 3.2 kV, cone voltage at 25 V, collision energy at 18 eV (sofalcone) and 15 eV (sofalcone metabolite), respectively, and dwell time of 0.30 sec. The peak widths of precursor and product ions were maintained at approximately 0.5 mass unit at half-height.

Sample preparation. All sample transfer and protein precipitation processes were automated by utilizing Perkin-Elmer MultiPROBE II HT (Boston, USA) and TOMTEC Quadra 96 workstations. Plasma specimens (0.2 mL) were transferred into 96-well plate by the automated sample handling system (MultiPROBE II HT), and spiked with 10 μL of 2 μg/mL d_3-sofalcone and d_3-sofalcone metabolite solutions (internal standard), respectively, using a Quadra 96 workstation (Tomtec Inc., Hamden, USA). 0.5 mL of acetonitrile were added to the plasma samples and then vortex mixed for 30 sec. After centrifugation, the supernatant was transferred into another 96-well plate and evaporated completely under a stream of nitrogen maintained at 40 °C. Dry residues were reconstituted with 120 μL of mobile phase consisted of acetonitrile/2 mM ammonium acetate, pH 6.15 (80 : 20; v/v). The 96-well plate was loaded onto the autosampler and then 20 μL of the reconstituted sample was injected into the LC-MS/MS system for analysis.

Validation procedures and calibration curves. Five replicate analyses were performed on plasma standards at five different concentration levels (2, 10, 50, 200, and 1000 ng/mL) of sofalcone and its metabolite, respectively, to assess both interday and intraday precision and accuracy of the method. The accuracy was expressed as [(mean observed concentration)/(spiked concentration)] × 100 (%), with the precision expressed as relative standard deviation (RSD). For preparation of analytical standards in plasma, appropriate amount (400 μL) of working solutions at different concentration levels (0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 μg/mL) were added to 50 mL polypropylene tubes containing 39.6 mL of pooled human blank plasma to yield final concentrations of 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL, respectively. The analytical standards were then vortex mixed, and 1.5 mL aliquots were transferred to 2 mL microcentrifuge tubes and stored at −70 °C until analysis. Analytical standards were used to construct the calibration curve.

Pharmacokinetic assay. For the human assay, a single 100 mg dose of sofalcone was administered orally to 26 volunteers who were advised about the nature and purpose of the study. The volunteers possessed good health and had not taken any medication for at least two weeks before the study. The group consisted of healthy males with a mean age of 23.7 ± 1.8, mean weight of 70.1 ± 6.2 kg, and mean height of 173.9 ± 4.8 cm. Blood samples were collected at 0, 0.33, 0.67, 1, 1.5, 2, 3, 4, 5, 6, 7 and 8 hr, respectively, after administration by using the heparin vacutainer collection tubes. Human plasma were obtained by centrifugation at 2000 *g* for 10 min. Plasma specimens were then stored at –70 °C until analysis.

Results and Discussion

Both sofalcone and its metabolite showed higher responses in positive ion electrospray ionization generating protonated molecular ions $[M+H]^+$ as base peaks. Sofalcone and its metabolite produced a protonated molecular ion ($[M+H]^+$) of *m/z* 451 and 453, respectively, and a corresponding product ion of *m/z* 315 and 317, respectively. Internal standards (d_3-sofalcone and d_3-sofalcone metabolite) produced a protonated molecular ion ($[M+H]^+$) of *m/z* 454 and 456 and a corresponding product ion of *m/z* 315 and 317, respectively. The product ion mass spectrum, and their postulated rationalization in terms of major fragmentation patterns, of sofalcone and its metabolite are illustrated in Figure 2(a) and Figure 2(b). The most abundant product ions (*m/z* 315 for sofalcone and *m/z* 317 for sofalcone metabolite)

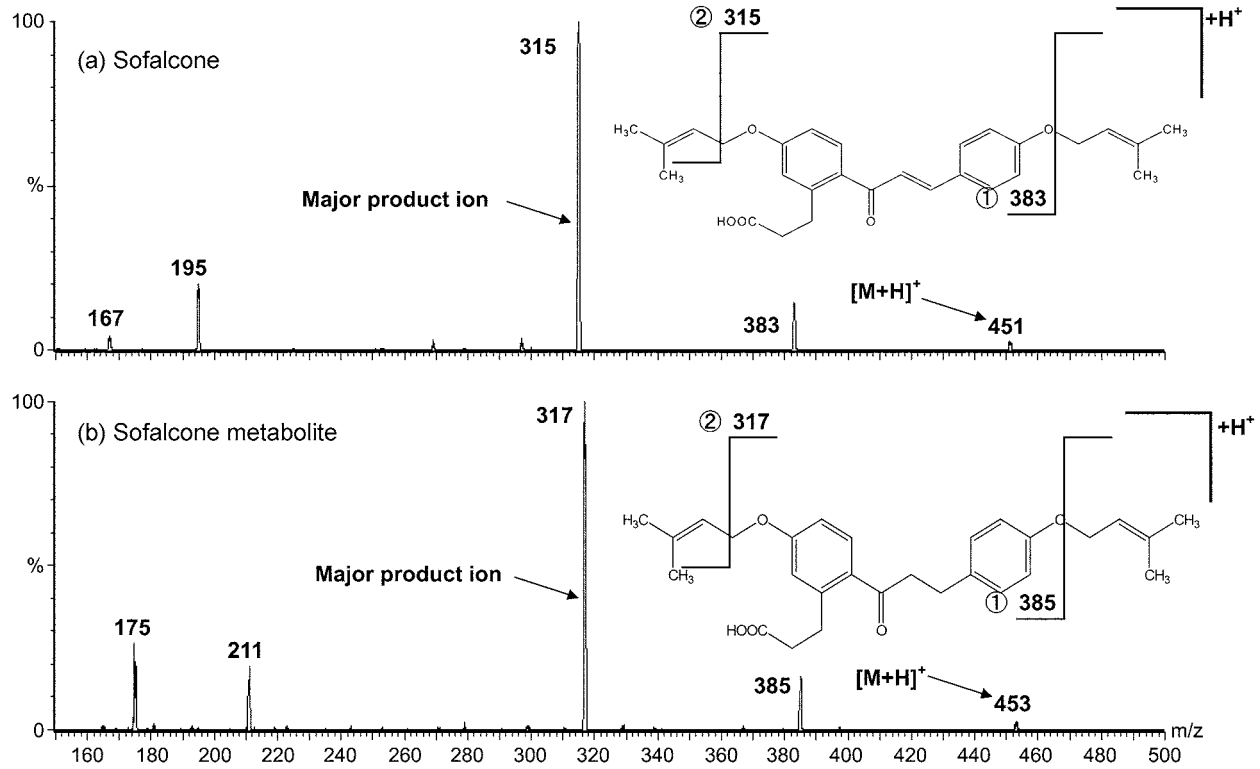

Figure 2. Product ion spectrum of (a) sofalcone and (b) sofalcone metabolite.

were selected for MRM analysis.

Choice of mobile phase, pH conditions, and flow rate still remain to be very important in order to achieve optimal sensitivity in LC-MS/MS. For sofalcone and its metabolite, we initially attempted to develop a reversed phase chromatographic method using our previous mobile phase conditions (80% acetonitrile: 20% 2 mM ammonium acetate, pH 6.15 with acetic acid)[4] and were able to successfully obtain sensitive mass spectrometric data of sofalcone and its metabolite without observing any matrix effects. However, retention time of sofalcone and its metabolite was very similar. The retention time of sofalcone and its metabolite were 1.92 min and 1.87 min, respectively. Therefore, we attempted to achieve better chromatographic separation of sofalcone and its metabolite by using a gradient. Mobile phase A was consisted of acetonitrile (pH 6.15 with acetic acid) and mobile phase B was consisted of 2 mM ammonium acetate (pH 6.15 with acetic acid). The initial mobile phase composition (40% A) was found to be crucial for a better separation of sofalcone and its metabolite, as a result retention time has increased. Higher initial organic composition (> 80%) causes co-elution of sofalcone and its metabolite, and a lower composition (< 40%) cause generated long retention time. Therefore, a gradient (from 40% to 80% A) combined with high column temperature was employed, resulting in better chromatographic separation for simultaneous characterization of sofalcone and its metabolite (Figure 3).

The stability of sofalcone and its metabolite was evaluated in the dissolution solvent and in human plasma. It was found that sofalcone and its metabolite were stable for the entire duration of the experiment.

The limit of quantitation (LOQ), defined as having a signal-to-noise ratio (S/N) of 10, of sofalcone and its metabolite was found to be 2 ng/mL by injecting 20 μL of sample into the LC-MS/MS system.

Sensitivity and selectivity of the method were evaluated by analyzing blank plasma from five different batches of pooled human blank plasma. Blank plasma both with and without internal standard was analyzed on each validation day. No interference was observed neither in the blank plasma with nor without internal standard. Figure 4 shows the mass chromatogram of the human blank plasma analyzed.

Since chromatographic conditions may cause co-elution of a number of endogenous compounds that are undetected by the MS/MS but can still affect the ionization efficiency, the matrix effect on the response of the analyte was also evaluated. In this effort, plasma samples from different sources (subjects) (n=5) were extracted and then spiked with the same amount of sofalcone and its metabolite. The final concentration of sofalcone and its metabolite was 50 ng/mL, respectively. The MS/MS responses and precision were then compared, with the same analysis repeated (n=5) after spiking a single source of matrix. If the responses and precision vary among different sources or within a single source, then the matrix effect exists. Otherwise, there is no matrix effect with similar responses and precision. No matrix effect was detected in this investigation (Table 1).

Analyte recovery assays were carried out both for sofalcone and its metabolite at five different concentration levels: 2, 10, 50, 200, and 1000 ng/mL, respectively. The concentration of standards spiked into blank matrix before

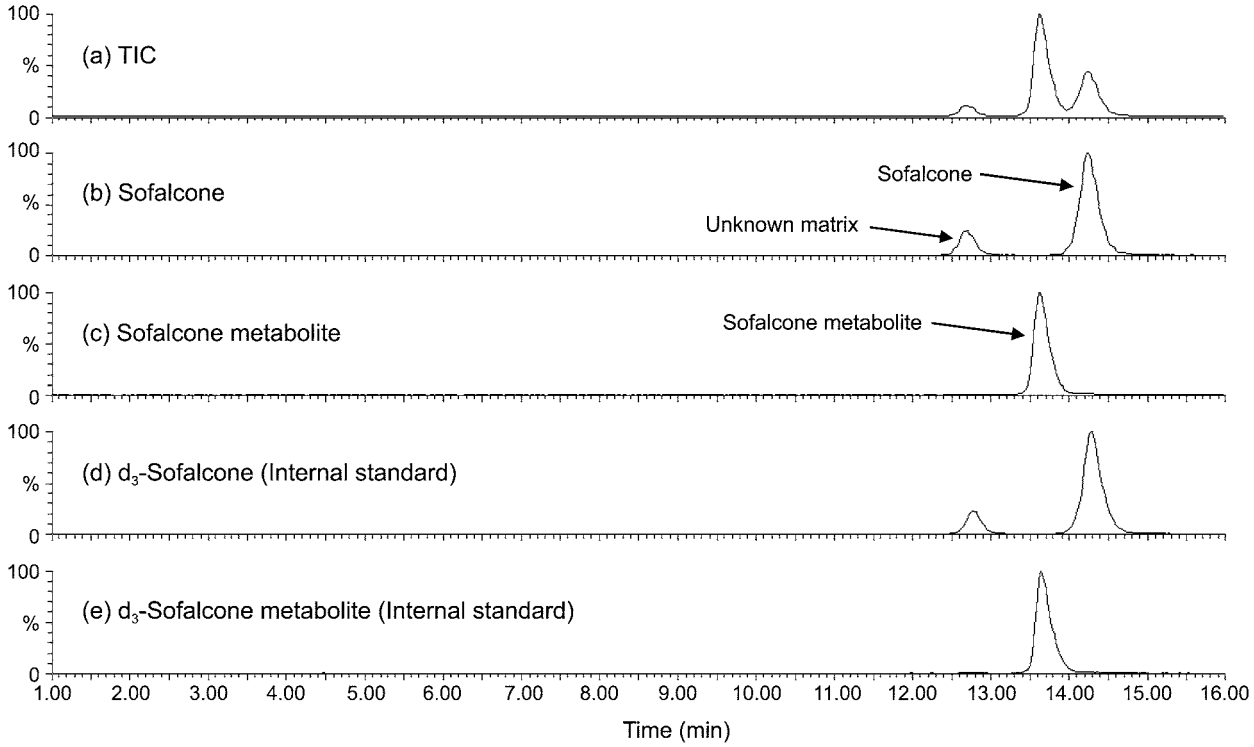

Figure 3. LC-MS/MS chromatograms of (a) TIC, (b) sofalcone (MRM: 451 → 315), (c) sofalcone metabolite (MRM: 453 → 317), (d) d_3-sofalcone (MRM: 454 → 315) and (e) d_3-sofalcone metabolite (MRM: 456 → 317) in human plasma.

Table 1. Analyte/IS response and precision of sofalcone and its metabolite for the matrix effect test

No	Sofalcone (Concentration : 50 ng/mL)		Sofalcone metabolite (Concentration : 50 ng/mL)	
	Different Source[a] (Analyte/IS Response)	Single Source[a] (Analyte/IS Response)	Different Source[a] (Analyte/IS Response)	Single Source[a] (Analyte/IS Response)
1	5.50E-02	5.39E-02	5.24E-02	5.21E-02
2	5.45E-02	5.41E-02	5.31E-02	5.11E-02
3	5.21E-02	5.47E-02	5.22E-02	5.39E-02
4	5.33E-02	5.27E-02	5.27E-02	5.28E-02
5	5.29E-02	5.29E-02	5.16E-02	5.25E-02
Mean	5.36E-02	5.37E-02	5.24E-02	5.25E-02
SD	1.18E-03	8.41E-04	5.61E-04	1.02E-03
% RSD	2.21	1.57	1.07	1.95

[a]Source = Human plasma

extraction was compared to the concentration of standards spiked into blank matrix after extraction for recovery calculations. Measurements at each concentration level were conducted in five replicates. As a result, sofalcone and its metabolite showed recovery levels of 86.7-91.1% and 86.1-89.8%, respectively (Table 2).

The intraday precision expressed in % RSD was determined to be 2.74-5.21% (sofalcone) and 1.70-6.47% (sofalcone metabolite) at by performing five replicate analyses at each concentration level. The intraday accuracy expressed in percentage of nominal values was determined to be 99.0-105.5% (sofalcone) and 99.8-104.0% (sofalcone metabolite) at the same five concentration levels by performing five replicate analyses at each concentration level. Table 3 shows the intraday precision and accuracy of measurement of

Table 2. Recovery of sofalcone and its metabolite in human plasma

Standard concentration in plasma (ng/mL)	Recovery[a] (%) of sofalcone	Recovery[a] (%) of sofalcone metabolite
2	91.1	89.8
10	87.3	86.1
50	88.2	89.3
200	86.7	87.4
1000	87.9	87.7

[a]Five replicates at each concentration level (n=5)

sofalcone and its metabolite in human plasma. The interday precision was determined to be 3.07-7.73% (sofalcone) and 2.28-7.35% (sofalcone metabolite) at 2, 10, 50, 200, and 1000 ng/mL by performing five replicate analyses at each

Table 3. Intraday precision and accuracy of measurement of sofalcone and its metabolite in human plasma

Nominal Concentration (ng/mL)	Sofalcone [Mean ± SD][a] Calculated Concentration (ng/mL)	Accuracy (%)	Precision (%RSD)	Metabolite [Mean ± SD][a] Calculated Concentration (ng/mL)	Accuracy (%)	Precision (%RSD)
2	2.11 ± 0.11	105.5	5.21	2.01 ± 0.13	100.5	6.47
10	10.1 ± 0.5	101.0	4.95	10.4 ± 0.6	104.0	5.77
50	50.1 ± 1.7	100.5	3.39	51.4 ± 1.6	102.8	3.11
200	198 ± 6	99.0	3.03	202 ± 7	101.0	3.47
1000	1021 ± 28	102.1	2.74	998 ± 17.	99.8	1.70

[a]Five replicates at each concentration level (n=5)

Table 4. Interday precision and accuracy of measurement of sofalcone and its metabolite in human plasma

Nominal Concentration (ng/mL)	Sofalcone [Mean ± SD][a] Calculated Concentration (ng/mL)	Accuracy (%)	Precision (%RSD)	Metabolite [Mean ± SD][a] Calculated Concentration (ng/mL)	Accuracy (%)	Precision (%RSD)
2	2.07 ± 0.16	103.5	7.73	2.04 ± 0.15	102.0	7.35
10	10.0 ± 0.7	100.0	7.00	10.3 ± 0.7	103.0	6.80
50	48.9 ± 2.8	97.8	5.73	49.9 ± 2.1	99.8	4.21
200	201 ± 8	100.5	3.98	197 ± 9	98.5	4.57
1000	1011 ± 31	101.1	3.07	1008 ± 23	100.8	2.28

[a]Five replicates at each concentration level (n=5)

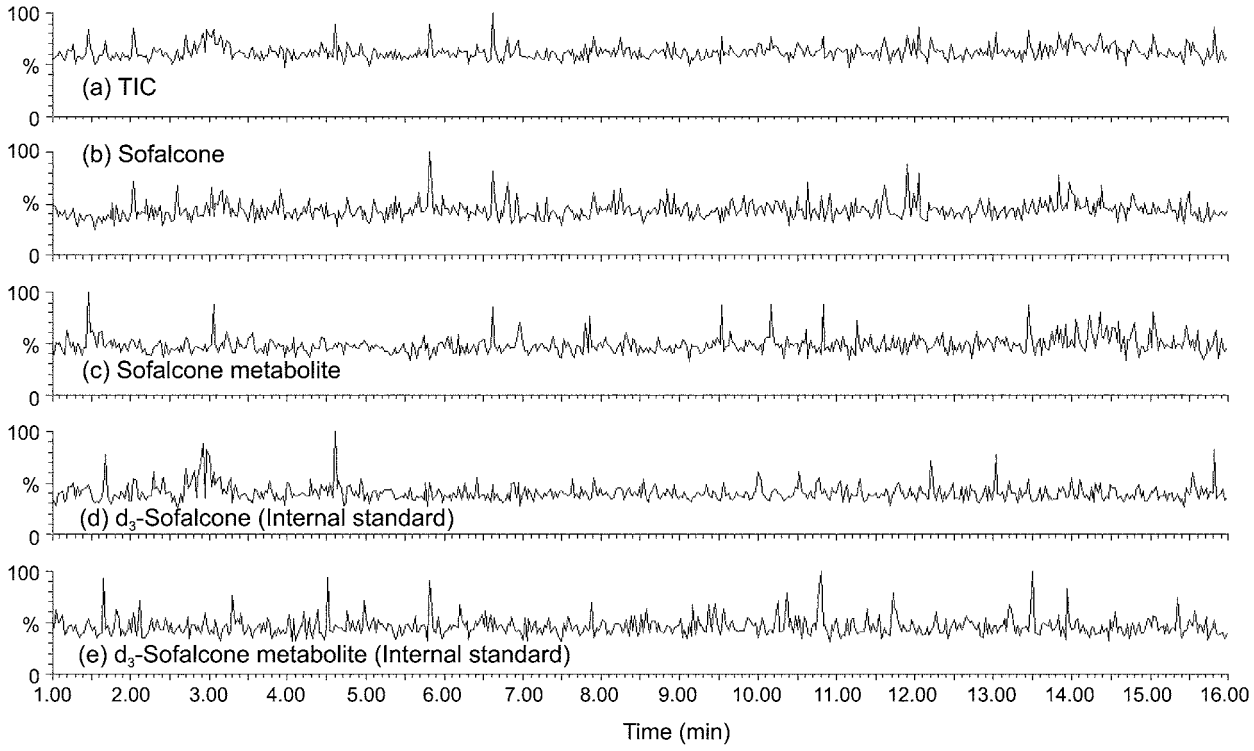

Figure 4. LC-MS/MS chromatograms of blank human plasma without internal standard.

concentration level. The interday accuracy was determined to be 97.8-103.5% (sofalcone) and 98.5-103.0% (sofalcone metabolite) at the same five concentration levels by performing five replicate analyses at each concentration level. Table 4 shows interday precision and accuracy of measurement of sofalcone and its metabolite in human plasma.

Calibration standard curves (y = mx + b) were generated by using a weighted linear least-squares regression of the peak area ratios (y) of the analytes to their internal standards versus the concentrations (x) of the calibration standards. Concentrations of analytes in QC samples were back calculated using the resulting peak area ratios and the regression equations of the calibration curves. Analytical standards and QC samples were prepared freshly on each different working day (10 days) in order to construct calibration curves. A good linear response was obtained

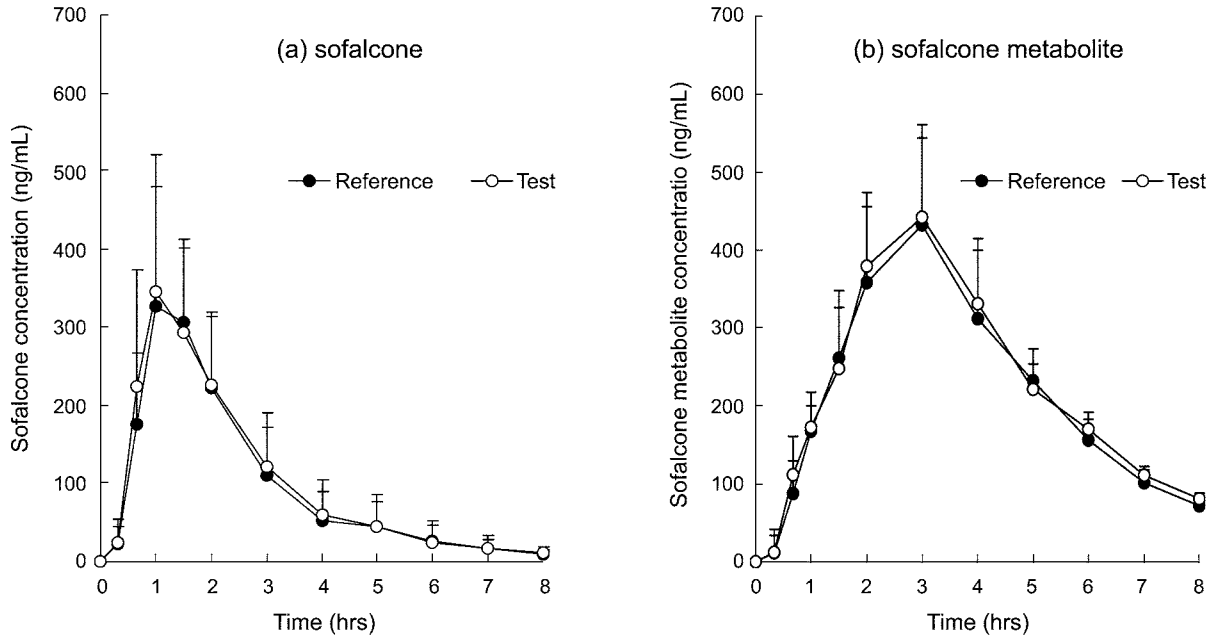

Figure 5. Plasma concentration of (a) sofalcone and (b) its metabolite in human plasma - time curve.

Table 5. Pharmacokinetic parameters of reference and test sofalcone

No		Reference			Test		
		AUC[a] (ng/mL/h)	C_{max}[b] (ng/mL)	t_{max}[c] (h)	AUC (ng/mL/h)	C_{max} (ng/mL)	t_{max} (h)
Mean ± SD	Sofalcone	723.9 ± 186.7	389.1 ± 99.8	1.19 ± 0.27	759.7 ± 203.8	381.2 ± 112.0	1.17 ± 0.30
	Sofalcone Metabolite	1760 ± 441	432.1 ± 112.3	3.02 ± 0.47	1826 ± 486	442 ± 118.9	3.03 ± 0.52

[a]AUC: Area under the plasma concentration – time curve extrapolated to infinity. [b]C_{max}: Peak plasma concentration. [c]T_{max}: Time to reach C_{max}

throughout the dynamic range of the study, with an r^2 value always greater than 0.9997. Calibration curves for sofalcone and its metabolite have shown that the assay has good reproducibility over a wide dynamic range of 2-1000 ng/mL.

The validated method was applied to determine concentrations of sofalcone and its active metabolite in human after a single oral administration of a 100 mg sofalcone tablet to 26 healthy volunteers. Figure 5(a) and Figure 5(b) show the concentration of sofalcone and its metabolite in human plasma at each time point, respectively. Table 5 compares the pharmacokinetic parameters of reference sofalcone and its metabolite with those of the test sofalcone and its metabolite. These results indicate that our new assay offers outstanding selectivity, sensitivity, and reproducibility. In summary, we report a novel assay developed for pharmacokinetic studies to determine the concentration of sofalcone and its metabolite in human plasma simultaneously.

Conclusion

A novel method for the determination of sofalcone and its active metabolite using automated sample preparation and LC-MS/MS has been developed and validated, with a lower quantitation limit of 2 ng/mL. Validation experiments have shown that the assay has good precision and accuracy over a wide dynamic range (2-1000 ng/mL), and no interferences caused by endogenous compounds were observed by the matrix effect test. Automated sample preparation significantly reduces sample preparation time, increases sample throughput, and improves the assay reproducibility. This simple and robust assay has the capacity to handle efficient analysis of a large number of samples that is essential for pharmacokinetic studies of sofalcone and its metabolite in human plasma.

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