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Quantification of sofalcone in human plasma and urine by high performance liquid chromatography–mass spectrometry

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ABSTRACT

Sofalcone, isolated from the root of the Chinese medicinal plant *Sophora subprostrata*, is well known to be a mucosal protective agent for gastritis and peptic ulcer treatment. Although the LC–MS/MS and HPLC-DAD methods for assay of plasma concentration of sofalcone were reported before for the pharmacokinetic study, they were either too complicated or not sensitive enough for current pharmacokinetic study. In addition, no urinary assay method or pharmacokinetic information was available. Thus an improved high performance liquid chromatography–mass spectrometric method employing negative electrospray ionization was developed for the determination of sofalcone concentration in human plasma and urine sample. A liquid–liquid extraction method was utilized to extract sofalcone together with the indometacin (internal standards) from 0.5 ml of human plasma or urine samples. Multiple reaction monitoring was used for quantification by monitoring the transition of m/z from 449.5 to 313.1 for sofalcone and 356.9 to 313.0 for IS. The validation of the method regarding to specificity, sensitivity, linearity, reproducibility, accuracy and stability was evaluated. The lower limit of quantification (LLOQ) of the developed assay method for sofalcone was 0.5 ng/ml and the linear calibration curve was acquired with $R^2 > 0.99$ between 0.5 and 500 ng/ml for both plasma and urine samples. The intra- and inter-day variations of the current assay were evaluated with the relative standard deviation (RSD) within 13.77% at low concentration of quality control samples (QCs) and 8.71% for other QCs, whereas the mean accuracy ranged from 96.21 to 107.33%. The samples were found to be stable under the storage conditions at least for a month and other experimental conditions. This validated method was then utilized to test sofalcone concentration in clinical samples. Based on these data, the pharmacokinetic behavior of sofalcone in plasma as well as urine was described. As a conclusion, the present method proved to be a rapid and sensitive analytical tool for sofalcone in human plasma or urine samples and has been successfully applied to a clinical pharmacokinetic study of in healthy Chinese subjects.

1. Introduction

Sofalcone, 2′-carboxymethoxy-4,4′-bis(3-methyl-2-butenyloxy) chalcone, is a type of flavonoid and a synthetic derivative of sophoradine which is isolated from the root of the Chinese medicinal plant *Sophora subprostrata* [1,2]. The drug is well known to be a mucosal protective agent to treat patients with gastritis and peptic ulcer in China, Japan and South Korea [3]. The pharmacological efficacy of sofalcone is due to the inhibition of 15-hydroxy-prostaglandin (PG)-dehydrogenase, or increasing blood flow in mucosa. In addition, it also exhibits the anti-inflammatory effects on *H. pylori*-associated gastritis via inhibiting of pro-inflammatory cytokine production [4].

The pharmacokinetic behavior of sofalcone has been performed in human subjects including Chinese and Korea populations [5,6]. When given orally, sofalcone can be absorbed rapidly from gastrointestinal tract with the T_{max} around 1 h. It was found to be metabolized into its alkane metabolite by hydrogenation. However, there is no information of the renal clearance of sofalcone in human body. The HPLC-DAD and LC–MS/MS method for plasma concentration determination was reported before [5,6], but both of them were far from optimization. The HPLC-DAD method was simple but with a compromised sensitivity (LLOQ: 20 ng/ml), while the HPLC–MS/MS method was quite sensitive but the sample processing was complicated and only can be applied with aid of special instrument. Moreover, a relatively long run time (16 min) was required for the analysis. Thus an improved HPLC–MS/MS assay method was developed utilizing the HPLC–MS/MS with liquid–liquid extract method to detect sofalcone in both plasma and urine samples.

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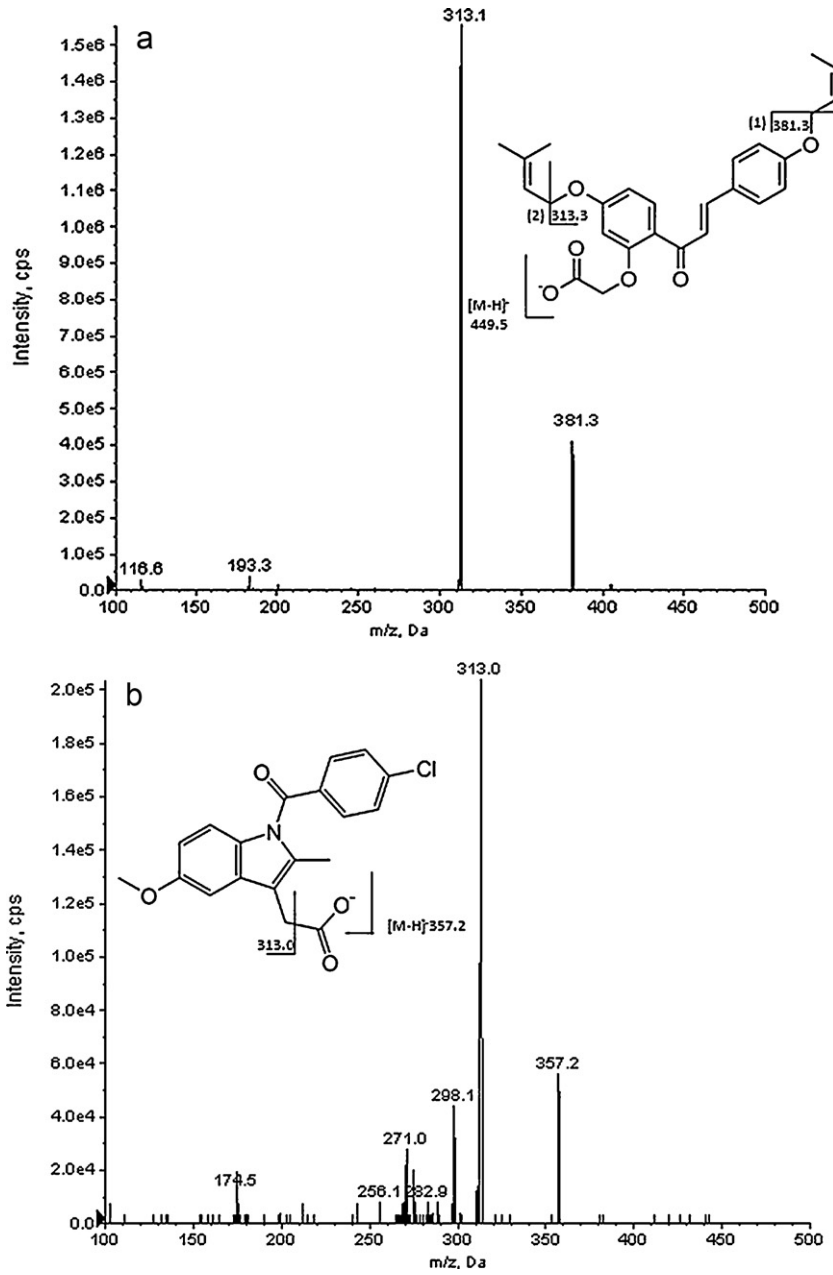

Fig. 1. Product ion mass spectra of (a) sofalcone and (b) indometacin.

2. Materials and methods

2.1. Chemicals and reagents

Sofalcone standard was provided by Xinyi Co., Ltd. (Shanghai, China). Indometacin (internal standard, IS), formic acid and ammonium acetate of analytical grade were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA) with purity greater than 99%. HPLC grade methanol, acetonitrile (ACN), ethyl acetate (EA) and n-hexane were got from Merck (Darmstadt, Germany). The deionized water was distilled in our lab using a Millipore AFS-10 water purification system (Millipore, Billerica, MA, USA). Blank plasma and urine were supplied by West China Hospital, Sichuan University (Chengdu, China). The pharmaceutical preparation of sofalcone (100 mg) capsule was obtained from the Taisho Pharmaceutical Co., Ltd (Solon®, Japan).

2.2. Preparation of calibration curve and QC samples

One mg of sofalcone and indometacin were accurately weighted and dissolved in 1 ml of methanol to prepare the stock solution. The two stock solutions and subsequent working solutions were stored at 4 °C and were tested to be stable for at least 3 months.

The working solutions of sofalcone with concentrations of 5, 10, 20, 100, 200, 500, 1000, 2000, 4000, 5000 ng/ml were prepared by diluting the stock solution appropriately using 50% methanol. Calibration standards were prepared by spiking 50 μl of working solutions into 500 μl of blank plasma or urine samples. The calibration ranges were over 0.5–500.0 ng/ml for both plasma and urine. Four levels of QC samples were chosen as LLOQ, low, medium and high calibration standard concentrations, i.e. 1.0 ng/ml, 20.0 ng/ml and 400.0 ng/ml. The QC samples were stored at −20 °C with clinical sample to be analyzed.

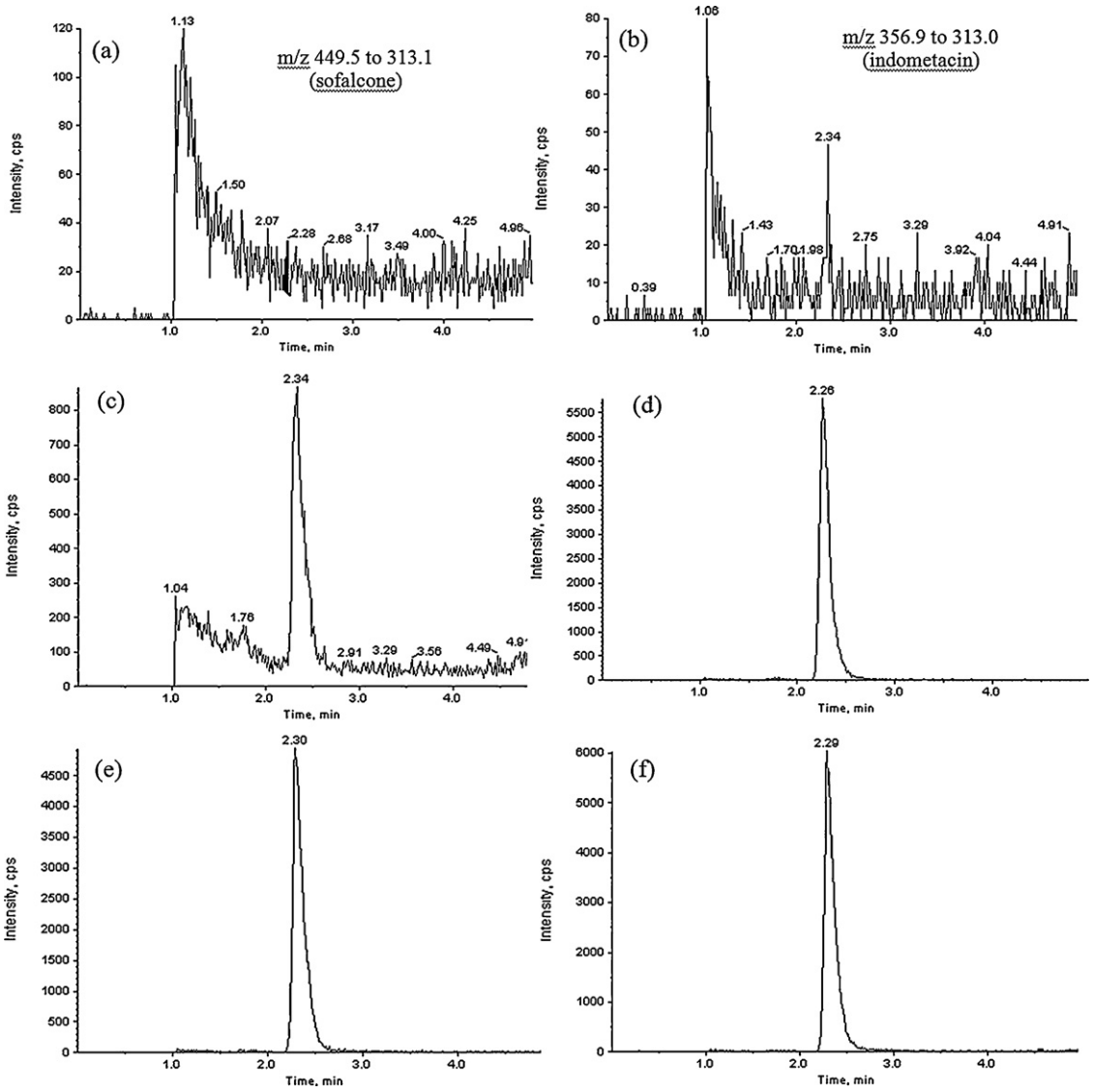

Fig. 2. (A) MRM chromatogram of (a) and (b): blank plasma; (c) and (d) 0.5 ng/ml of sofalcone with its IS (100 ng/ml); (e) and (f) a plasma sample obtained from a subject at 6 h post dosing (concentration determined was 4.3 ng/ml) with its IS (100 ng/ml). (B) MRM chromatogram of (a) and (b) blank urine sample; (c) and (d) 0.5 ng/ml of sofalcone with its IS (100 ng/ml); (e) and (f) a urine sample obtained from a subject (concentration determined was 30.8 ng/ml) with its IS (100 ng/ml).

2.3. Sample preparation

For analysis of the real clinical samples, 50 μl of 50% methanol was spiked instead of the corresponding working solutions as mentioned above. Samples were then homogenized by vortexing briefly. Liquid–liquid extraction was then performed by addition of 4 ml of an n-hexane/EA (3:7, V/V) mixture, followed by vortex extraction for 10 min (Vortex Genie® 2 Vortex, Carlsbad, CA, US). After centrifugation at 12,000 rpm for 5 min, the upper organic layer was transferred into another tube and completely evaporated to dryness at 40 °C under a stream of nitrogen (Turbovap Zymark, Hopkinton, MA, USA). The dry residue was reconstituted with 100 μl of mobile phase. After centrifugation at 12,000 rpm for 5 min, a 20 μl supernatant was injected into the LC–MS/MS system.

2.4. Instrumentation and chromatographic conditions

LC–MS/MS analysis was performed on an Agilent 1100 HPLC system (Palo Alto, CA, USA) coupled to an API 3200 mass spectrometer (Applied Biosystems, Foster City, CA, USA). The LC system was equipped with an Xterra® MS C_{18} column (3.5 μm, 2.1 mm × 150 mm) and a Xterra® MS C_{18} (3.5 μm, 2.1 mm × 10 mm) guard column. The column temperature was set at 35 ± 2 °C. An isocratic elution was carried out using a mobile phase containing 75% ACN and 25% formic acid (0.2%, containing 2 mM ammonia acetate). The flow rate was 0.4 ml/min.

The LC eluent for the first 2 min was set to bypass the MS system. Afterwards, the eluent was monitored in the negative ionization mode by switching the 10-port valve from waste to the mass spectrometer. Typical mass spectrometric conditions were as follows: curtain gas, 25 psi; ionization spray voltage, −5500 V; gas temperature (TEM), 480 °C; turbo ion spray gas (gas 1), 30 psi, heater gas (gas 2), 40 psi. Multiple reaction monitoring (MRM) scanning was used to identify the molecules by monitoring the transition of *m*/*z* from 449.5 to 313.1 for sofalcone and 356.9 to 313.0 for IS respectively. Analyst (version 1.5) software was used for instrument control and quantification.

2.5. Validation procedures

The validity of the assay method was assessed according to FDA "Guidance for Industry – Bioanalytical Method Validation" protocol

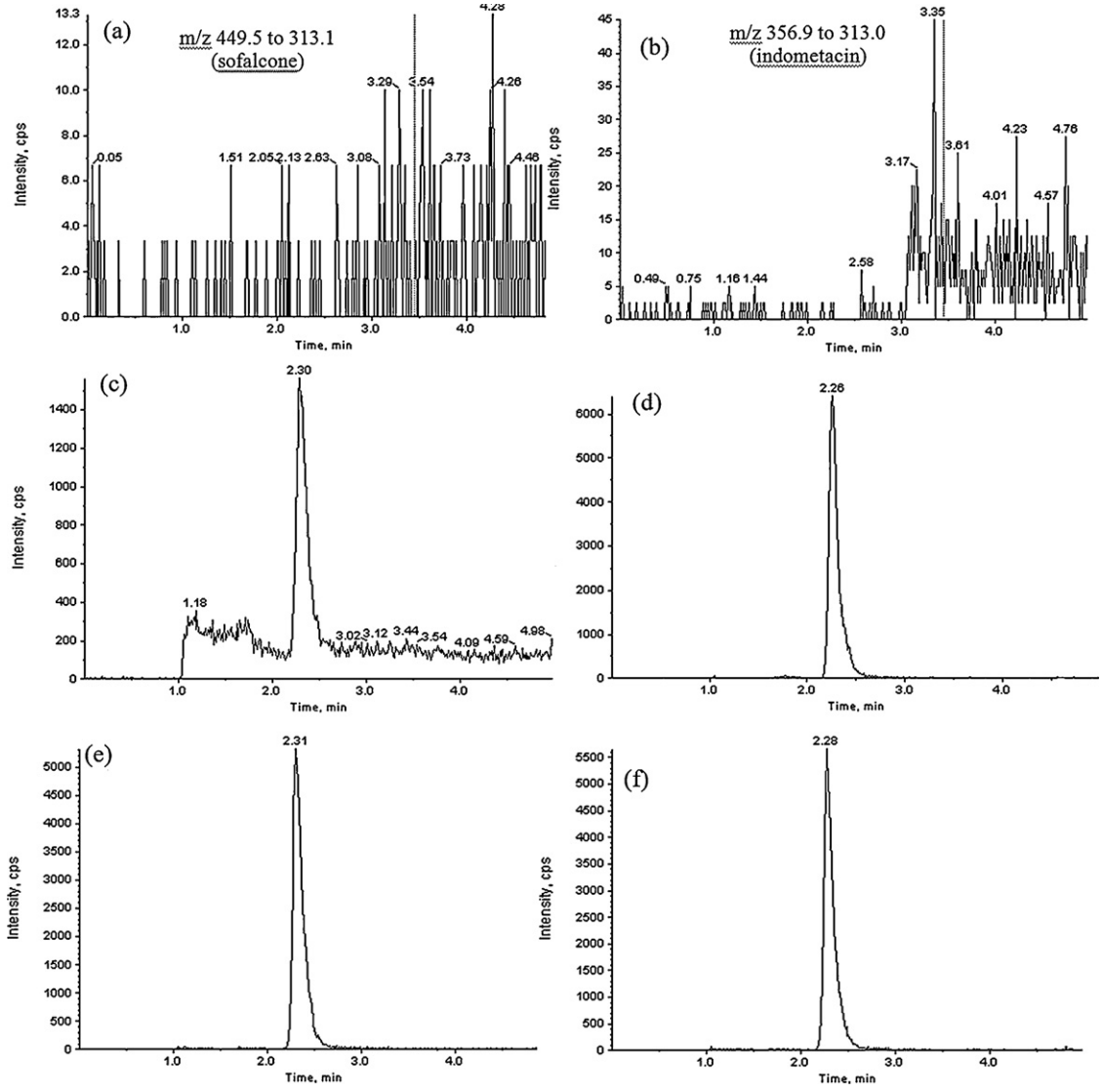

Fig. 2. (*Continued*).

in terms of the linearity, sensitivity, precision, accuracy, recovery, LLOQ and stability [7]. Matrix effects were evaluated as outlined by Matuszewski et al. [8].

2.5.1. Specificity and selectivity

Endogenous interference was determined by the analysis of blank plasma and urine samples originating from 4 different sources. Blank matrix samples spiked only with IS or sofalcone were analyzed to assess potential interference that may affect either the sofalcone or IS.

2.5.2. Sensitivity and linearity

Calibration curves were obtained by plotting the peak area ratio of the analyte and IS against their corresponding concentrations. Calibration was performed using weighted linear regression with factor of 1, $1/x$, or $1/x^2$. The LLOQ was determined based on (1) the analyte response should be at 5 times that of the blank response and (2) analyte peak response should be identifiable, discrete, and reproducible with a precision of 20% and accuracy within 80–120%.

2.5.3. Precision and accuracy

To assess the intra-day precision and accuracy of the method, the plasma and urine QCs (0.5, 1, 20 and 400 ng/ml) were analyzed in five replicates in a single run, while for inter-assay variation, the QCs samples were analyzed in three separate days. The precision was defined as the relative standard deviation (RSD) of the determined concentrations of the same QCs, whereas accuracy was assessed as the percentage to the nominal concentration (%). The mean values should be within 15% of the nominal value except at LLOQ, which should not deviate by 20%.

2.5.4. Stability

Short-term bench top stability of sofalcone in plasma and urine was determined by analysing three aliquots of each QC samples after thawing and storage for 4 h at room temperature. Freeze thaw stability was evaluated after three freeze and thaw cycles, i.e. three aliquots of each of the QC samples in plasma and urine, stored at −20 °C, were thawed unassisted at room temperature and refrozen for at least 12 h. This cycle was repeated two more times. On the third cycle, QCs were analyzed with the freshly prepared QCs. Autosampler stability was assessed by re-analyzing the same sample after being stored in autosampler (4 °C) for 12 h. Long-term stability was estimated by assessing the QCs sample stored at −20 °C for 1 month. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (within 15%).

Table 1
Precision and accuracy for determination of sofalcone in plasma and urine.

Matrix	Nominal conc. (ng/ml)	Intra-day determination Determined conc. (ng/ml)	Precision (%)	Accuracy (%)	Inter-day determination Determined conc. (ng/ml)	Precision (%)	Accuracy (%)
Plasma	0.5	0.45 ± 0.09	19.98	90.20	0.48 ± 0.09	18.75	95.90
	1	0.99 ± 0.12	12.12	99.20	1.02 ± 0.14	13.73	102.25
	20	19.24 ± 1.42	7.38	96.21	21.24 ± 1.85	8.71	106.18
	400	409.09 ± 22.73	5.56	102.27	403.17 ± 20.52	5.09	100.79
Urine	0.5	0.43 ± 0.07	16.28	86.01	0.51 ± 0.10	19.61	102.30
	1	0.93 ± 0.13	13.98	93.20	1.07 ± 0.04	3.74	107.33
	20	21.14 ± 1.60	7.57	100.68	20.22 ± 1.60	7.91	101.08
	400	407.76 ± 21.00	5.15	101.94	390.97 ± 19.33	4.94	97.74

Table 2a
Extraction recovery of sofalcone and indometacin in plasma.

	Conc. (ng/ml)	% Recovery ($n = 3$)	% Matrix effect				
			Absolute matrix effect ($n = 12$)	Source 1 ($n = 3$)	Source 2 ($n = 3$)	Source 3 ($n = 3$)	Source 4 ($n = 3$)
Sofalcone	1	82.77	100.69	105.39	109.02	89.19	99.15
	20	76.92	99.59	106.94	103.23	90.60	97.59
	400	80.67	96.99	94.32	95.96	101.35	96.35
Indometacin	100	85.29	99.07	110.95	89.13	93.10	103.10

2.5.5. Extraction recovery and matrix effect

When mass spectrometer was used as detector, a matrix effect by ionization competition between the analytes and co-eluents may exist. The potential presence of a matrix effect for sofalcone and IS was evaluated in 4 different sources of biological fluid (plasma and urine). The absolute matrix effect (%) was calculated according to the following equation.

$$\text{Matrix effect (\%)} = \frac{\text{Peak area of standards spiked post extraction}}{\text{Peak area of standards spiked in neat mobile phase}} \times 100\%$$

Peak area at each concentration was compared between the different sources of biological fluid to evaluate the relative matrix effect.

Extraction recovery was determined by calculating the peak area ratio of the analyte at the same concentration levels, spiked before and after extraction. Recovery (%) was determined as:

$$\text{Recovery (\%)} = \frac{\text{Peak area for standards spiked before extraction}}{\text{Peak area for standards spiked post extraction}} \times 100\%$$

This value represents a recovery value that is not affected by the matrix.

2.6. Pharmacokinetic assay

The bioanalytical method developed here was applied to clinical samples collected from 24 healthy male subjects after oral dose of 100 mg sofalcone. The study was performed in accordance with the principles of the Declaration of Helsinki and approved by the independent Ethics Committee of Sichuan University and consistent with Good Clinical Practice guidelines. Volunteers between 18 and 30 years old (24.05 ± 2.71) with a body mass index of 18–24 participated in this open-label study. All of them provided gave their written informed consent for participation in the study. After fasting 10 h, participants orally received 100 mg sofalcone with 200 ml of water. Venous blood samples (3.5 ml) were immediately collected into heparinized tubes before dose and after dose at 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 15 and 24 h. Urine samples were also collected 0–4, 4–8, 8–12 and 12–24 h. The total volume of urine collected at each time intervals were measured and recorded. Ten milliliter of each urine sample was retained and stored at −20 °C until analysis.

Plasma and urine were harvested by centrifuging the blood and urine samples at 8000 rpm for 5 min and stored frozen at −20 °C until analysis.

Pharmacokinetic parameters including *AUC*, $t_{1/2}$, k_e, C_{max}, and T_{max} were calculated by a non-compartmental analysis using WinNonlin (version 2.1; Pharsight Corporation, Mountain View, CA). The cumulative amount of sofalcone ($X_{u,0-24}$) excreted in urine was calculated directly from the urinary concentration and volume. The total renal clearance (CL_r) was calculated as:

$$CL_r = \frac{X_{u,0-24}}{AUC_{0-24}}$$

3. Results and discussion

3.1. Specificity, sensitivity and linearity

The full Q1 scan of sofalcone and indometacin was acquired by negative ion mode using ESI source. Their product ion mass spectra are shown in Fig. 1.

The representative chromatograms of sofalcone and indometacin in both plasma and urine samples are shown Fig. 2(A) and (B). The typical retention times were 2.31 and 2.29 min for sofalcone and indometacin respectively. No interference from the endogenous plasma or urine was observed surrounding the two compounds. The sofalcone and its alkane metabolite have very similar chemical properties as per their structures as reported [5] and chromatographic separation could be difficult. Thus using UV detector may generate unreliable results, while MRM scanning can easily get the two compounds resolved, e.g. ligustrazine was reported to be accurately detected in herbal extract by MRM scanning rather than UV detection [9]. In addition, this method is characteristic with a very short running time (5 min) in comparison to reported methods (more than 15 min) and a reduction of solvent consumption.

The lower limit of quantification (LLOQ) was 0.5 ng/ml for both the plasma and urine samples. A good linearity was observed for the plasma samples as well as urine samples over a concentration range of 0.5–500 ng/ml (R^2 = 0.998, 0.997 for plasma and urine sam-

Table 2b
Extraction recovery of sofalcone and indometacin in urine.

	Conc. (ng/ml)	% Recovery ($n = 3$)	% Matrix effect				
			Absolute matrix effect ($n = 12$)	Source 1 ($n = 3$)	Source 2 ($n = 3$)	Source 3 ($n = 3$)	Source 4 ($n = 3$)
Sofalcone	1	84.08	100.53	90.86	102.23	102.23	106.81
	20	79.83	101.53	108.17	98.42	100.05	99.48
	400	81.50	103.86	107.17	109.78	95.70	102.81
Indometacin	100	83.83	96.33	88.24	96.04	97.42	103.62

ples respectively) with the weighting factor of $1/x^2$ (x is the nominal concentration) found to be best fit for both the low and high concentrations. In this study, liquid–liquid extraction was started from 500 μl of plasma or urine samples and eventually reconstituted in 100 μl volume. Thus the sensitivity was increased compared to the reported LC–MS/MS method (0.5 vs. 2 ng/ml) due to the 5:1 concentrating process [5]. In this clinical study, around 50% of plasma samples from 15 to 24 h were low than 2 ng/ml. Although increasing dose can lead to higher plasma concentration, it will cause unnecessary high drug exposure to subjects. The higher sensitivity of the assay method was practical to the pharmacokinetic study of sofalcone with sampling time up to 24 h even at regular or low oral dose.

3.2. Precision and accuracy

The intra- and inter-day precision and accuracy for the determination of sofalcone in plasma and urine are summarized in Table 1. For plasma samples the intra- and inter-day precision were within 12.02% and 13.77% respectively except for the LLOQ (within 19.98%), with accuracy ranging from 90.20 to 106.18%. For urine samples at concentrations of the intra- and inter-day precision were 13.73% and 7.91% respectively except for the LLOQ (within 19.61%), and the accuracy was ranged from 86.01 to 107.33%.

3.3. Matrix and recovery

The absolute matrix effect was within the range of 96.33–103.86%, which suggested there was no significant matrix effect on the signal intensity of both sofalcone and indometacin by the endogenous substance. For the relative matrix effect, the analyte's response was consistent from the samples collected from 4 different individuals (see Table 2a. Thus the method was considered to be valid, due to the similar relative matrix effect and unobvious absolute matrix effect.

The current extraction method yielded an average recovery of 80.1% and 81.8% for sofalcone in plasma/urine samples. The mean extraction recovery was 85.3% and 83.8% for IS in plasma and urine respectively (Table 2b).

3.4. Stability

Sofalcone was found to be stable in both plasma and urine samples for at least three freeze–thaw cycles or after storage at −20 °C for 1 month. No significant degradation of sofalcone was observed when the extracts were kept in the auto-sampler for up to 12 h at 4 °C.

3.5. Pharmacokinetics

The validated method was applied to determine plasma and urine concentrations of sofalcone in human subjects after single oral dose of a 100 mg sofalcone capsule. The plasma concentration–time profiles is shown in Fig. 3 with the corresponding pharmacokinetic parameters listed in Table 3.

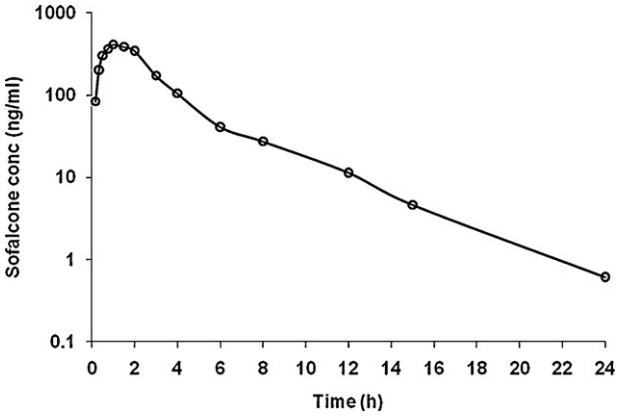

Fig. 3. Mean plasma concentration–time course in 24 subjects following an oral dose of 100 mg of sofalcone.

Table 3
Mean pharmacokinetic parameters of sofalcone in 24 subjects following an oral dose of 100 mg sofalcone capsule.

Parameters	Mean	Standard deviation
T_{max} (h)	1.14	0.26
C_{max} (ng/ml)	413.5	87.8
AUC_{0-24} (μg h/ml)	1.38	0.66
$T_{1/2}$ (h)	2.90	0.63

C_{max}: Peak drug concentration, obtained directly from the original concentration–time data.
T_{max}: Time to peak drug concentration, obtained directly from the original concentration–time data.
AUC_{0-24}: Area under the concentration–time curve from time zero to the last sampling time 24 h, calculated using log linear trapezoidal rule.
$T_{1/2}$: Terminal elimination half-life, calculated as $0.693/\lambda z$, where is the elimination rate calculated using the semi-log linear regression from the terminal phase of concentration–time curve.

The recovery of sofaclone over 24 h was 251.3 μg (0.25% of the total amount) after receiving 100 mg of sofalcone. The mean renal clearance was 182.1 ml/h. These results indicated very small amount of the parent drug was excreted from the kidney. The biotransformation should be the major pathway for elimination of sofalcone from human body, while the renal clearance contributes little to the total clearance.

4. Conclusion

These results indicated that our new assay offered a rapid assay method with outstanding selectivity, sensitivity, and reproducibility for concentration determination of solfacone in both plasma and urine samples. This method has been successfully applied to a clinical sofalcone pharmacokinetic study.

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