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Determination of four pyridine alkaloids from *Tripterygium wilfordii Hook. f.* in human plasma by high-performance liquid chromatography coupled with mass spectrometry

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ABSTRACT

A novel liquid chromatography–atmospheric-pressure chemical ionization–mass spectrometry (LC–APCI/MS) method was developed and validated for the simultaneous determination of four sesquiterpene pyridine alkaloids (wilfortrine, wilfordine, wilforgine and wilforine) in human plasma. The chromatographic separation was performed on a Shim-pack XR-ODS column using an ammonium acetate buffer solution–acetonitrile in a gradient program. The detection was achieved by an ion trap mass spectrometry in the positive selected ion monitoring (SIM) mode. The method utilized acetonitrile as protein precipitation solvent and followed by solid-phase extraction (SPE). Calibration curves were linear for the four alkaloids over the range of 0.5–100.0 μg/L with the limits of quantification of 0.5 μg/L, while the method exhibited the recovery of 86.5–98.6%, intra- and inter-day RSDs of less than 8.2% and 12.8%, respectively. Methodology was validated in line with the EU requirements (Commission Decision 2002/657/EC). Results of incurred samples demonstrated excellent reproducibility. To our knowledge, this is the first analytical method for simultaneous determination of the four sesquiterpene pyridine alkaloids in plasma. The method was applicable to clinical pharmaceutical research of alkaloids in rheumatoid arthritis volunteer patients after oral administrations.

1. Introduction

Sesquiterpene pyridine alkaloids are a large group of highly oxygenated sesquiterpenoids, all that based on a core C_{15} skeleton known as dihydro-β-agarofuran. They are considered to be the chemotaxonomic indicators of the family *Tripterygium wilfordii Hook. f.* (TWHF), which has been used as a traditional Chinese medicine for hundreds of years [1]. In the last 20 years, many dihydro-β-agarofuran sesquiterpene alkaloids, such as wilfortrine, wilfordine, wilforgine and wilforine [2–6] (as shown in Fig. 1), have been isolated mainly from TWHF plants. Modern pharmacologic researches found that the sesquiterpene pyridine alkaloids have immunosuppressive, antitumor-promoting and cytotoxic, antiviral, and anti-inflammatory properties [7]. Recently, some of these sesquiterpene pyridine alkaloids have been of great interest due to their cytotoxicity against several human tumour cell lines [8], insect antifeedant and insecticidal activities [9,10], and immunosuppressive activities [10–13]. Interestingly, the exciting biological activity of sesquiterpene pyridine alkaloids was proved to have anti-AIDS activities, such as wilfortrine showed anti-HIV activity ($IC_{50} > 100$ μg/mL, $EC_{50} < 0.1$ μg/mL, therapeutic index (TI) > 1000), and wilfordine showed anti-HIV activity ($IC_{50} = 20$ μg/mL, $EC_{50} < 0.1$ μg/mL, therapeutic index (TI) > 200) [14–16]. For the clinical study on sesquiterpene pyridine alkaloids pharmacokinetics, a sensitive and specific analytical assay was required to determine concentrations of sesquiterpene pyridine alkaloids in plasma.

So far, many methods have been developed for the determination of diterpenoid triepoxides from TWHF in different complicated matrix [17–24], however only a few reports are known for the analysis of the bioactive sesquiterpene pyridine alkaloids. To the best of our knowledge, there is only an ultraviolet (UV) spectrophotometry method for determination of the total TWHF alkaloids [25], a liquid chromatography (LC) method and a capillary eletrophoresis (CE) method both with an UV detector for the qualification of wilforine in TWHF extracts [26–28]. Unfortunately, the UV method lacks sufficient sensitivity and the CE method is difficult to implement due to the lack of popularity in China. Although Ouyang

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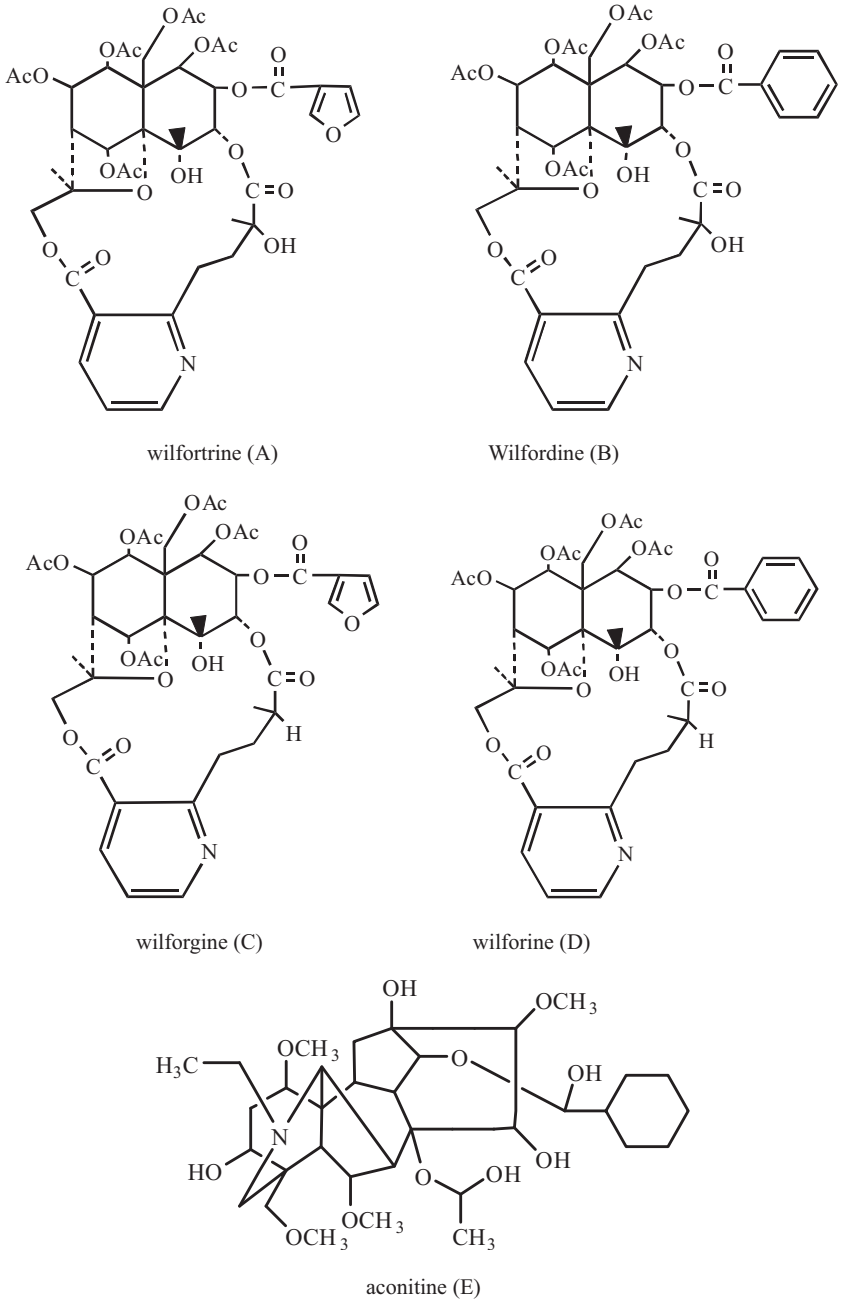

Fig. 1. Chemical structures of (A) wilfortrine with the molecular formula $C_{41}H_{47}NO_{20}$ and molecular weight 873 Da; (B) wilfordine with the molecular formula $C_{43}H_{49}NO_{19}$ and molecular weight 883 Da; (C) wilforgine with the molecular formula $C_{41}H_{47}NO_{19}$ and molecular weight 857 Da; (D) wilforine with the molecular formula $C_{43}H_{49}NO_{18}$ and molecular weight 867 Da and (E) aconitine (IS) with the molecular formula $C_{34}H_{47}NO_{11}$ and molecular weight 645 Da.

et al. [28] proposed an assay for the simultaneous determination of wilfortrine, wilfordine, wilforgine and wilforine in TWHF extracts by LC–UV, the extract matrix without lipids and proteins were less complicated than the human plasma. Therefore, further study on the analysis of the main sesquiterpene pyridine alkaloids, such as wilfortrine, wilfordine, wilforgine and wilforine, in plasma is still necessary. Mass spectrometry (MS) has played an important role in pharmaceutical analysis due to its sensitivity, rapidity, and low levels of sample consumption for the characterization and determination [29–36]. Especially, in combination with LC, it is considered a superior alternative to LC–UV or LC–FLD for the analysis of many natural active compounds. Although more than 50 sesquiterpene pyridine alkaloids have been isolated from TWHF plants, a few analytical methods were developed for the determination of the alkaloids in different biological matrices like plasma, serum and urine. The aim of this work is to develop and validate a rapid, sensitive and specific liquid chromatography–atmospheric-pressure chemical ionization–mass spectrometry (LC–APCI–MS) method using aconitine (Fig. 1) as an internal standard (IS) for the simultaneous determination of wilfortrine, wilfordine, wilforgine and wilforine in human plasma.

2. Experimental

2.1. Chemicals and solvents

Acetonitrile and acetic acid of HPLC grade were purchased from Merck (Darmstadt, German). Ammonium acetate used for

mobile phase was of HPLC-grade obtained from Sigma (Steinheim, Germany), while Oasis® MCX SPE cartridge used for sample preparation was procured from Waters (Milford, MA, USA). Deionized water was obtained using a Milli-Q water purification system from Millipore (Molsheim, France). The standards of wilfortrine, wilfordine, wilforgine and wilforine were isolated from a TWHF crude extract by chromatographic techniques in our laboratory [37]. Identification of the isolated compounds was confirmed by HPLC–MS, ^{1}H NMR and ^{13}C NMR methods. Purity of wilfortrine, wilfordine, wilforgine and wilforine were >98.5%, as determined by HPLC with a UV detector. Aconitine (>99.5%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

The six blank human plasma sources were collected from six healthy volunteers, aged from 19 to 23 years old, who were from Ningbo University (Ningbo, Zhejiang, China). Samples A–E were collected from five volunteer patients who were treated in Ningbo Traditional Chinese Medical Hospital (Ningbo, Zhejiang, China) for the disease of rheumatoid arthritis, and had taken the TWHF tablets for more than two days. A physical examination for the five volunteer patients was performed, and written informed consent forms were obtained before initiation of the TWHF alkaloids analysis study. The route, dose and time of administration of the study were defined according to the clinical doctor's advice. The study protocol and informed consent forms were approved by the ethics committee of Ningbo Municipal Center for Disease Control and Prevention.

2.2. Liquid chromatography–mass spectrometry

The HPLC separation for method development and validation was carried out on an Agilent 1100 series system, consisting of a quaternary pump, a column thermostat, a degasser unit, an autosampler, and an Agilent LC–MSD Trap SL mass spectrometer with an APCI interface (Agilent Technologies, Germany). The LC–APCI–MS system was controlled, and data were analyzed, on a computer equipped with LC–MSD Trap Software 4.2 (Bruker Daltonics, Bremen, Germany).

The chromatographic separation was performed on a Shim-pack XR-ODSII column (100 mm × 2.0 mm i.d., 2.2 μm, Shimadzu Corporation, Kyoto, Japan), using a buffer solution/acetonitrile as a mobile phase with a gradient elution program at a constant flow rate of 0.30 mL/min. The autosampler temperature was set at 4 °C. The column was maintained at a constant temperature of 35 °C in column oven and the injection volume was 20.0 μL. A gradient elution program was used to separate the four alkaloids and aconitine (IS) from the bulk of the endogenous matrix components. Mobile phase A was a buffer solution of 5 mmol/L ammonium acetate in 0.2% (v/v) acetic acid solution. Mobile phase B consisted of acetonitrile/5 mmol/L ammonium acetate–0.2% acetic acid buffer solution (90/10, v/v). The solvent gradient program was as follows: from 0 to 3.0 min, a linear gradient for mobile phase A was from 70% to 5%; from 3.0 to 4.0 min, mobile phase A was held at 5%; from 4.0 to 4.2 min, a linear gradient for mobile phase A was from 5% to 70%; from 4.2 to 7.0 min, mobile phase A was held at 70%.

The mass spectrometer equipped with an APCI was run in positive ion mode. The scan range was set to *m/z* 200–1000 using a cycle time of 1 s, a corona current of 4.0 μA, a capillary voltage of 3.1 kV, a capillary exit voltage of +118 V, a dry temperature of 350 °C, a vaporizer temperature of 450 °C, a high purity (99.999%) dry nitrogen gas of 8.0 L/min, a nitrogen nebulizer pressure of 60.0 psi and a dwell time of 200 ms. The target compounds were readily assignable in selected ion monitoring (SIM) mode, the ions of $[M+H]^+$ observed in the mass spectra, were *m/z* $[M+H]^+$874, *m/z* $[M+H]^+$884, *m/z* $[M+H]^+$858, *m/z* $[M+H]^+$ 868, and *m/z* $[M+H]^+$645 for wilfortrine, wilfordine, wilforgine, wilforine and aconitine (IS), respectively.

2.3. Preparation of standard stock solutions

Wilfortrine, wilfordine, wilforgine, wilforine and IS standards were accurately weighed, transferred to volumetric flasks and dissolved in acetonitrile/water (70:30, v/v) to make individual stock solutions of 1.0 g/L. These solutions were thoroughly mixed and stored at 4 °C in tightly closed bottles until use. Interim standard solutions of 10.0 mg/L for wilfortrine, wilfordine, wilforgine, wilforine and IS were diluted from the stock solutions (1.0 g/L) with acetonitrile/water (70:30, v/v). A series of working standard solutions were diluted from the interim standard solutions (10.0 mg/L) in acetonitrile/water (70:30, v/v) at a level of 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, 20.0, 50.0 and 100.0 μg/L for each analyte, wherein the IS concentration was 10.0 μg/L.

2.4. Preparation of spiked human plasma samples

The working solutions of wilfortrine, wilfordine, wilforgine and wilforine (500 μL) was transferred into a series of 2 mL polypropylene centrifuge tubes, and evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted with 500 μL of blank human plasma, which was thawed to room temperature in advance, to give final wilfortrine, wilfordine, wilforgine and wilforine concentrations of 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, 20.0, 50.0 and 100.0 μg/L, wherein the IS concentration was 10.0 μg/L. These sequences of spiked human plasma solutions were considered as the matrix-matched calibration standards, and the four concentration levels of 0.5, 2.0, 15.0 and 50.0 μg/L of wilfortrine, wilfordine, wilforgine and wilforine in human plasma were considered as quality control (QC) samples. The QC samples and blank human plasma were stored in freezer at −20 °C and completely thawed prior to use. Each batch of calibration standards was freshly prepared and stored at 4 °C until the initiation of LC–MS analysis (not more than 20–30 min).

2.5. Preparation of sample

The blank human plasma samples were thawed and homogenized. Using a micropipette, 500 μL plasma and 5.0 μL IS (1.0 mg/L) were transferred into a 2.0 mL polypropylene centrifuge tube before analysis, extracted using 1.0 mL acetonitrile and vortex-mixed by Vortex-Genie-2 mixer for 5 min. After centrifugation for 5 min at 7800 rpm, the upper organic layer was transferred to a disposable glass tube and the extraction was re-operated once. The combined organic layers were evaporated to dryness under a stream of nitrogen on a heating block at 50 °C in a disposable glass tube. Afterwards, the residues were dissolved in 2.0 mL water/acetonitrile (40/60, v/v), and the tube was immersed in a KQ 500DB ultrasonic cleaning bath (Kunshan Ultrasonic, Jiangsu, China) and ultrasonicated for 2.0 min to facilitate dissolution. Then, the sample was loaded on a MCX cartridge previously conditioned with 1.0 mL acetonitrile and followed by 1.0 mL water. The cartridge was washed with 2.0 mL water, followed by 1.0 mL water/acetonitrile (25/75, v/v). Alkaloids and IS were eluted with 1.0 mL acetic acid/acetonitrile (0.2/99.8, v/v). The eluate (20 μL) was directly injected into the HPLC–MS in partial loop mode. After concentrating samples to dryness, they were dissolved in acetonitrile/water (70:30, v/v) prior to LC–MS analysis.

2.6. Method validation

2.6.1. Specificity

To investigate whether the presence of endogenous constituents would interfere with the assay, six blank human plasma samples were analyzed to detect any potential interference due to co-eluting with the analytes and IS. Chromatographic peaks of the

analytes and IS were identified by comparing their retention time and SIM responses with those of authentic standards.

2.6.2. Matrix effect

The investigation of the matrix effect on an atmospheric-pressure chemical ionization (APCI) was evaluated by comparative analyses on two sets of samples based on the approach proposed by Matuszewski et al. [38]. In the first set (A), three concentration levels (low, medium and high concentrations, 2.0, 15.0 and 50.0 μg/L, respectively) of neat standards were dissolved in acetonitrile and were analyzed in triplicates. Another set (B), blank human plasma homogenate samples which were free of any significant interferences at the retention time of analyte and IS, were extracted by SPE and then spiked with three levels of standards. The influence of endogenous components on LC–MS was calculated by comparing the ratio of peak areas of matrix sample after sample preparation with that of a neat standard. The matrix effect results obtained in this study were calculated as follows:

$$\text{matrix effect} = \left[1 - \frac{Y}{X}\right] \times 100\%$$

where X and Y represent the mean peak areas of alkaloid standards in acetonitrile/water (70:30, v/v) and spiked post-extraction at corresponding concentrations, respectively.

2.6.3. Precision and accuracy

Precision (expressed by relative standard deviation (RSD) for replicate measurements) and accuracy (expressed by the percentage of bias between nominal and calculated concentrations) were evaluated by analysis of six replicates of QCs at four concentration levels (0.5, 2.0, 15.0, 50.0 μg/L) for three successive batches. The intra-day precision of the method was checked by performing six replicates of QC samples containing four concentration levels (0.5, 2.0, 15.0, 50.0 μg/L) of the alkaloids within a day in order to assess the intra-day variability ($n = 6$). The inter-day precision was monitored by performing triplicates of the four spiked QC samples on eight different days within a 14-day period. The analytical results were compared with those of freshly prepared samples. RSD was taken as a measure of precision. The deviation of each concentration level from the nominal concentration was expected to be within ±15%. Similarly, the RSD of mean accuracy should not exceed 15% of the actual value.

The accuracy was expressed by the recoveries which were calculated by comparing the peak areas obtained from extracted samples spiked with known amounts of standard before extraction to those of the standard solutions spiked after extraction. Experiments were performed by five replicate determinations of each alkaloids at four nominal concentrations (0.5, 2.0, 15.0, 50.0 μg/L).

The sensitivity of the analytical procedure was expressed as the lower limit of quantification (LLOQ) or the lowest concentration on the calibration curve that can be quantitatively determined with acceptable accuracy within 80–120% and precision less than 20%. The signal to noise (S/N) of each analyte at LLOQ should be at least 10.

3. Results and discussion

3.1. Chromatography and mass spectrometry

A HPLC–MS system has been employed for the simultaneous determination of the four sesquiterpene pyridine alkaloids and IS in human plasma. In an attempt to develop a satisfactory separation of the target compounds, a Shim-pack XR-ODSII column (100 mm × 2.0 mm i.d., 2.2 μm) was chosen for the separation owing to the better performance including peak shape, peak intensity, and retention time at ambient temperature. After evaluating many different combinations of organic solvents and aqueous buffers, a mobile phase, composed of A (5.0 mmol/L ammonium acetate in 0.2% acetic acid buffer solution) and B (5.0 mmol/L ammonium acetate in 0.2% acetic acid buffer solution–acetonitrile) (10:90, v/v), was optimized in a gradient elution. This mobile phase gave the maximal peak intensity and best peak shape as well as a short retention time with a flow rate of 0.30 mL/min. The retention time of wilfortrine, wilfordine, wilforgine, wilforine and IS was 2.4, 4.9, 5.2, 4.3 and 5.8 min, respectively, with an overall chromatographic run time of 7 min (Fig. 2). Acetonitrile was used for protein precipitation, because of its good efficiency in precipitating and extraction and its compatibility with the mobile phase.

The MS parameters were optimized to produce an abundant area of wilfortrine, wilfordine, wilforgine, wilforine and IS, respectively, using an atmospheric-pressure chemical ionization. The highest signal to noise ratio was achieved in the positive mode. For all five compounds, their protonated molecular ions of m/z 874, 884, 858, 868 and m/z 646 were dominant and were therefore used for quantification. Fig. 2 shows the SIM chromatograms of the four alkaloids and IS obtained from a blank human plasma sample and a spiked human plasma sample. It can be seen that the employed gradient elution program produced a satisfactory separation of the four alkaloids and IS.

Aconitine was chosen as an internal standard as isotopically labelled alkaloids were not available, with which to calibrate the assay and to minimize errors related to the pretreatment of the samples. In fact, the recovery of the alkaloids after pretreatment varies, depending on the nature of the sample and the differences in pretreatment conditions used by various laboratories. Therefore, it is necessary to use an internal standard to calibrate the responses of the internal standard with that of the analyte in the determination. Fortunately, it was found that aconitine was suitable as an internal standard as it has relatively similar chromatographic properties to the four compounds and the same ionization properties, and recovery was similar. Representative chromatograms of spiked plasma samples and a blank sample are shown in Fig. 2.

3.2. Specificity

The specificity of the method was examined by analyzing ($n = 6$) blank human plasma extracted again plasma spiked with the lowest standard. Fig. 2 shows the representative chromatograms of a blank human plasma sample and a blank human plasma sample spiked with the four alkaloids (5.0 μg/L) and the IS (10.0 μg/L). No endogenous peaks at the retention times of wilfortrine, wilfordine, wilforgine, wilforine and IS were observed for any of the plasma samples. It indicated that there was no endogenous substance in plasma interfering in the analysis of alkaloids.

3.3. Matrix effect

The investigation of the matrix effect on the APCI was calculated by comparing the ratio of peak areas of matrix sample after sample preparation with that of a neat standard. Blank plasma samples were extracted, then spiked and analyzed for potential interferences by endogenous matrix components. Table 1 displays the data of matrix effect tests. At 2.0, 15.0 and 50.0 μg/L concentration levels, the analytes had matrix effects within the range of −5.8 to 4.6%. These indicate that there were no endogenous substances to influence significantly the ionization of the four analytes and IS.

3.4. Precision and accuracy

Table 2 shows the data of the intra- and inter-day precision. The results indicate that the intra-day precision (RSD) at

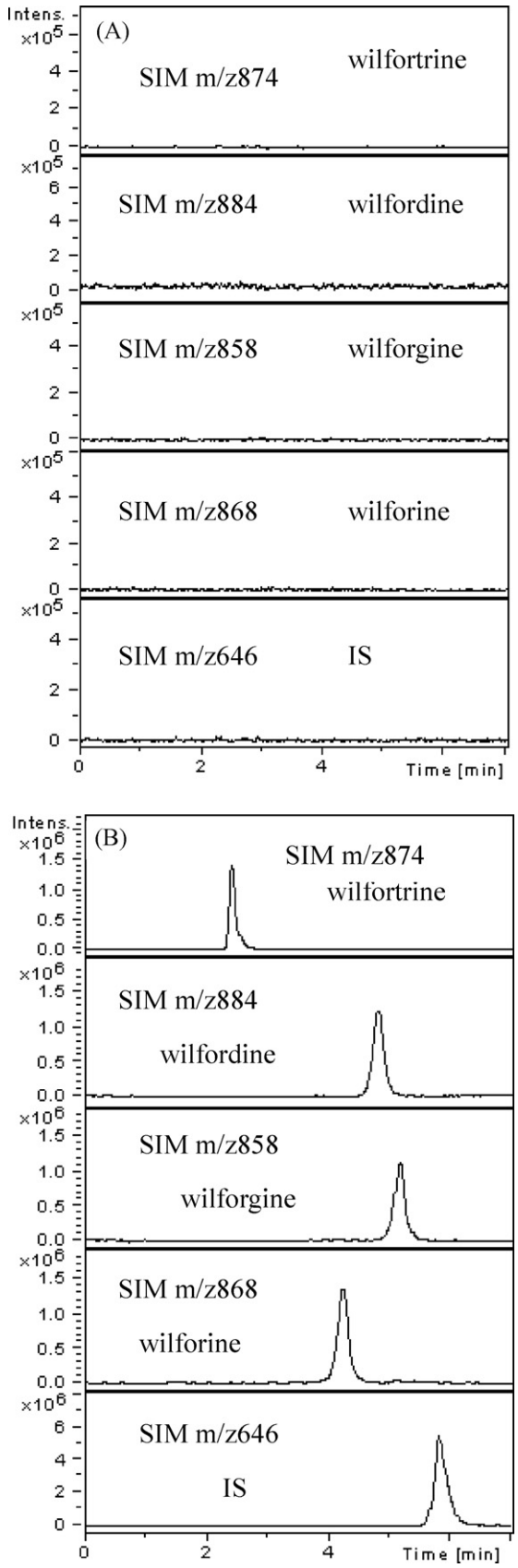

Fig. 2. SIM chromatograms for wilfortrine, wilfordine, wilforgine, wilforine and IS from human plasma extracts: (A) a blank plasma sample, (B) a blank plasma sample spiked with wilfortrine (5.0 μg/L), wilfordine (5.0 μg/L), wilforgine (5.0 μg/L), wilforine (5.0 μg/L) and the IS (10.0 μg/L).

Table 1
Calculated % matrix effects of plasma on the peak area response of alkaloids and IS (n = 5).

Compound	Concentration (μg/L)	Matrix effect (%)
Wilfortrine	2.0	−5.6
	15.0	−5.3
	50.0	4.6
Wilfordine	2.0	−4.3
	15.0	−4.6
	50.0	−5.8
Wilforgine	2.0	−5.5
	15.0	−4.9
	50.0	−3.5
Wilforine	2.0	−4.7
	15.0	−5.3
	50.0	−5.5
Aconitine (IS)	10.0	3.9

the four nominal concentrations (0.5, 2.0, 15.0, 50.0 μg/L) was between 6.3–7.4%, 4.7–8.2%, 4.8–6.5% and 5.2–6.8%, and the inter-day precision (RSD) the four nominal concentrations was between 8.2–12.8%, 8.1–12.4%, 6.8–9.8% and 8.3–11.9% for wilfortrine, wilfordine, wilforgine and wilforine, respectively.

The QC samples were prepared and analyzed together with the calibration samples. The extraction recovery (%) was calculated by comparing the mean peak areas of analytes spiked before extraction divided by the areas of analytes spiked after extraction and multiplied by 100. The results show that the mean recoveries of the alkaloids from human plasma samples (n = 6) at the four nominal concentrations were between 89.3–96.0%, 89.0–98.0%, 86.5–98.6% and 88.0–96.8% for wilfortrine, wilfordine, wilforgine and wilforine, respectively, as shown in Table 2.

3.5. Linearity, lower limit of detection (LLOD) and quantification (LLOQ)

The calibration curve was made to quantitate low, medium, and high alkaloids levels in human plasma. Calibrators were made by adding working stock solutions of alkaloids and IS to blank plasma for the final alkaloids concentrations of 0.5, 1.0, 2.0, 5.0, 15.0, 25.0, 50.0 and 100.0 μg/L with 10.0 μg/L IS. The linear calibration curves were plotted by internal standard method based on linear regression analysis of the integrated peak area ratios of analyte-to-IS (Y) versus the concentrations of analyte (C, μg/L). The typical regression equation obtained by least squared regression is shown in

Table 2
The recovery and the intra- and inter-day precision.

Compound	Added (μg/L)	Found[a] (μg/L)	Recovery (%)	RSD (%)	
				Intra-day[a]	Inter-day[b]
Wilfortrine	0.5	0.46 ± 0.03	92.0	6.5	12.8
	2.0	1.92 ± 0.12	96.0	6.3	8.8
	15.0	13.4 ± 0.99	89.3	7.4	10.9
	50.0	47.3 ± 3.2	94.6	6.8	8.2
Wilfordine	0.5	0.49 ± 0.04	98.0	8.2	12.4
	2.0	1.82 ± 0.11	91.0	6.1	11.7
	15.0	13.8 ± 0.65	92.0	4.7	8.1
	50.0	44.5 ± 3.0	89.0	6.0	8.9
Wilforgine	0.5	0.48 ± 0.03	96.0	6.3	9.8
	2.0	1.73 ± 0.10	86.5	5.7	8.9
	15.0	13.9 ± 0.67	92.7	4.8	7.7
	50.0	49.3 ± 3.2	98.6	6.5	6.8
Wilforine	0.5	0.44 ± 0.03	88.0	6.8	10.3
	2.0	1.85 ± 0.12	92.5	6.4	11.9
	15.0	14.1 ± 0.83	94.0	5.9	9.1
	50.0	48.4 ± 2.5	96.8	5.2	8.3

[a] n = 6, expressed as mean ± SD.
[b] n = 3 replicates × 8 days within a 14-day period, expressed as mean ± SD.

Table 3
The parameter for the developed LC–MS method.

Compound	Regression equation	Linear range[a] (μg/L)	Coefficient of determination (r^2)	Limit of detection[b] (μg/L)	Limit of quantification[b] (μg/L)	RSD of the slope and intercept[c] (%)
Wilfortrine	$C = 15.11Y - 0.42$	0.5–100.0	0.992	0.2	0.5	5.8 (5.0)
Wilfordine	$C = 14.57Y - 0.13$	0.5–100.0	0.996	0.2	0.5	5.1 (3.8)
Wilforgine	$C = 16.64Y - 0.67$	05–100.0	0.991	0.2	0.5	4.9 (5.4)
Wilforine	$C = 15.88Y - 0.88$	0.5–100.0	0.990	0.2	0.5	4.4 (6.8)

[a] Determined by the standard working solutions spiked at the human plasma.
[b] 500 μL of the human plasma.
[c] The RSD of the intercept is in the bracket.

Table 4
Assessment of stability in spiked blank plasma for the four alkaloids ($n = 3$).[a]

Compound	Added (μg/L)	Condition						
		Freeze–thaw stability (−20 °C)			Short-term stability (room temperature 25 °C)			
		Cycle 1	Cycle 2	Cycle 3	Time = 0.5 h	Time = 2.0 h	Time = 12.0 h	Time = 48.0 h
Wilfortrine	2.0	95.3	95.6	94.3	96.3	94.6	95.3	99.3
	15.0	95.9	90.6	95.7	94.7	97.2	93.2	91.6
	50.0	92.1	91.4	97.4	92.5	92.1	100.5	94.6
Wilfordine	2.0	92.4	97.4	96.3	94.6	95.3	95.8	94.3
	15.0	94.3	96.5	95.4	99.5	97.2	94.3	95.3
	50.0	95.3	92.1	94.2	103.5	95.0	99.3	96.8
Wilforgine	2.0	95.4	99.2	99.1	98.6	96.7	94.6	95.6
	15.0	96.5	96.4	99.2	97.5	98.5	102.5	99.6
	50.0	94.3	98.3	94.3	96.6	98.3	95.1	95.4
Wilforine	2.0	99.5	99.1	95.6	96.4	98.4	94.7	96.2
	15.0	100.5	95.4	97.7	98.4	98.2	98.9	99.8
	50.0	103.2	99.4	98.5	97.5	95.4	98.5	95.7

[a] Expressed as the mean concentration change percentage from time zero.

Table 3. The calibration curve was linear in the tested concentration range of 0.5–100.0 μg/L with a coefficient of determination (r^2) over 0.990 and consistent slope under 5.8% and intercept values under 6.8%.

LLOD was considered as the lowest concentration producing a signal-to-noise (S/N) ratio of no less than 3 and LLOQ was defined as the concentration producing S/N ratio of at least 10. The LLODs of this method were estimated to be lower than 0.2 μg/L for each alkaloid in human plasma, as shown in Table 3. The LLOQs of this method were 0.5 μg/L, at which level acceptable precision and accuracy were obtained, as shown in Table 2. This sensitivity of this method is better than those reported for previous HPLC methods [26–28], in which the LLODs were between 1.0 and 2.0 mg/L.

3.6. Stability

The freeze–thaw stability (three cycles, from −20 °C to the room temperature), and the room temperature stability (24 h in mobile phase) were studied by analyzing the three QC samples. There was no significant degradation (<9.4%) for the four alkaloids between the responses of standards at time zero and after undergoing freeze–thaw cycles for three times. These indicate that the four alkaloids were stable under this condition. A concentration stability of the four alkaloids was noted within 24 h when stored at room temperature, with no significant loss (<8.4%) at any QC concentrations, as shown in Table 4.

3.7. Application to the real samples

Samples A–E were analyzed by the above established HPLC–MS method. The measured concentrations for the four alkaloids in the patient plasma samples were 0–3.8 μg/L, and the wilfordine concentration was the highest in the plasma. The typical SIM chromatograms of the four alkaloids from a patient are showed in Fig. 3.

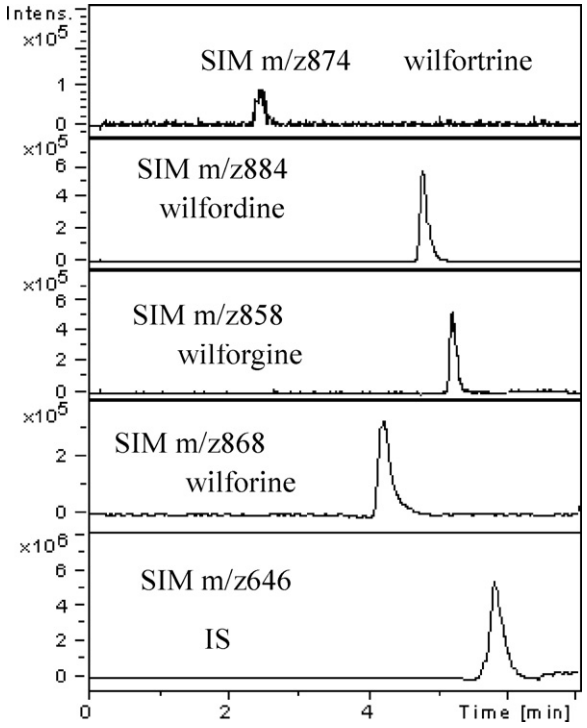

Fig. 3. Typical SIM chromatograms obtained from a patient.

From Fig. 3, it can be seen that there were no interferences for the determination of the four alkaloids in the real plasma samples.

4. Conclusions

In summary, the method is described for the quantification of wilfortrine, wilfordine, wilforgine and wilforine from human

plasma by LC–MS using the positive selected ion monitoring mode. The current method has shown acceptable precision and sensitivity for the quantification of the four alkaloids in human plasma. The validated method allows quantification of the four alkaloids in the 0.5–100.0 μg/L range, and it can be suitable for pharmacokinetic, bioavailability or bioequivalence studies.

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