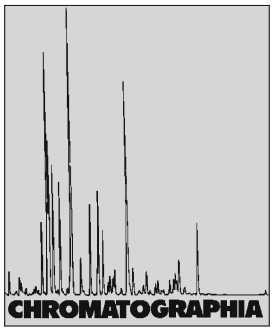
Simultaneous Quantification of Sodium Ferulate, Salicylic Acid, Cinnarizine and Vitamin B1 in Human Plasma by LC Tandem MS Detection



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Abstract

A rapid and simple high performance liquid chromatographic method coupled with tandem mass spectrometry (LC–MS–MS) via electrospray ionization (ESI) has been developed and validated to separate and simultaneously quantify sodium ferulate (SF), salicylic acid (SA), cinnarizine (CIN) and vitamin B1 (VB1) in human plasma. Gemfibrozil (GEM) was used as the internal standard (IS) for SF and SA, whereas lomerizine (LOM) was used as the IS for CIN and VB1. The plasma samples were prepared by one-step protein precipitation followed by an isocratic elution with 10 mM ammonium acetate buffer (pH = 5.0): acetonitrile (35:65, *v/v*,) on an Agilent Zorbax SB-CN column (150 mm × 2.0 mm ID, 5 μm). The precursor and product ions of these drugs were monitored on a triple quadrupole mass spectrometer, operating in the selected reaction monitoring mode (SRM) with polarity switch, in the negative-ion mode for SF, SA and GEM, in the positive-ion mode for CIN, VB1 and LOM. The method was validated over the concentration range of 1.5–1,000 ng mL^{-1} for SF, 20–5,000 ng mL^{-1} for SA, 2–500 ng mL^{-1} for CIN, 1–30 ng mL^{-1} for VB1. The intra- and inter-batch precisions were less than 15% of the relative standard deviation. The recoveries for analytes and IS achieved from spiked plasma samples were consistent and reproducible. The validated LC–MS–MS method has been successfully applied to the pharmacokinetic study of sodium ferulate and aspirin capsule in healthy volunteers.

Keywords

Column liquid chromatography
LC–MS–MS
Pharmacokinetics
Vitamin B1
Sodium ferulate
Salicylic acid
Cinnarizine

Introduction

Cardiovascular and cerebrovascular diseases are killing more and more people world wide. Those who survive a heart attack or stroke often need to take long-term medical treatment.

The compound of sodium ferulate and aspirin capsule containing 50 mg sodium ferulate (SF), 20 mg aspirin, 25 mg cinnarizine (CIN) and 10 mg vitaminB1 (VB1) is used for preventing and treating cardiovascular and cerebrovascular diseases in China. SF which is an effective component of Chinese medicinal herbs, such as *Angelica sinensis, Cimicifuga heracleifolia, and Ligusticum chuangxiong* has been found to inhibit platelet aggregation and metabolism of arachidonic acid, increasing coronary blood flow, relaxing or stimulating smooth muscle, possessing anti-arrhythmic, anti-oxidative, immunostimulating and anti-inflammatory effects, etc [1–4]. Aspirin inhibits platelet aggregation by irreversible inhibition of platelet cyclooxygenase and thus inhibiting the generation of thromboxane A_2, a powerful inducer of platelet aggregation and vasoconstriction [5]. CIN is used as an antihistaminic drug, calcium entry blocker for the treatment of cerebral and peripheral vascular insuffi-

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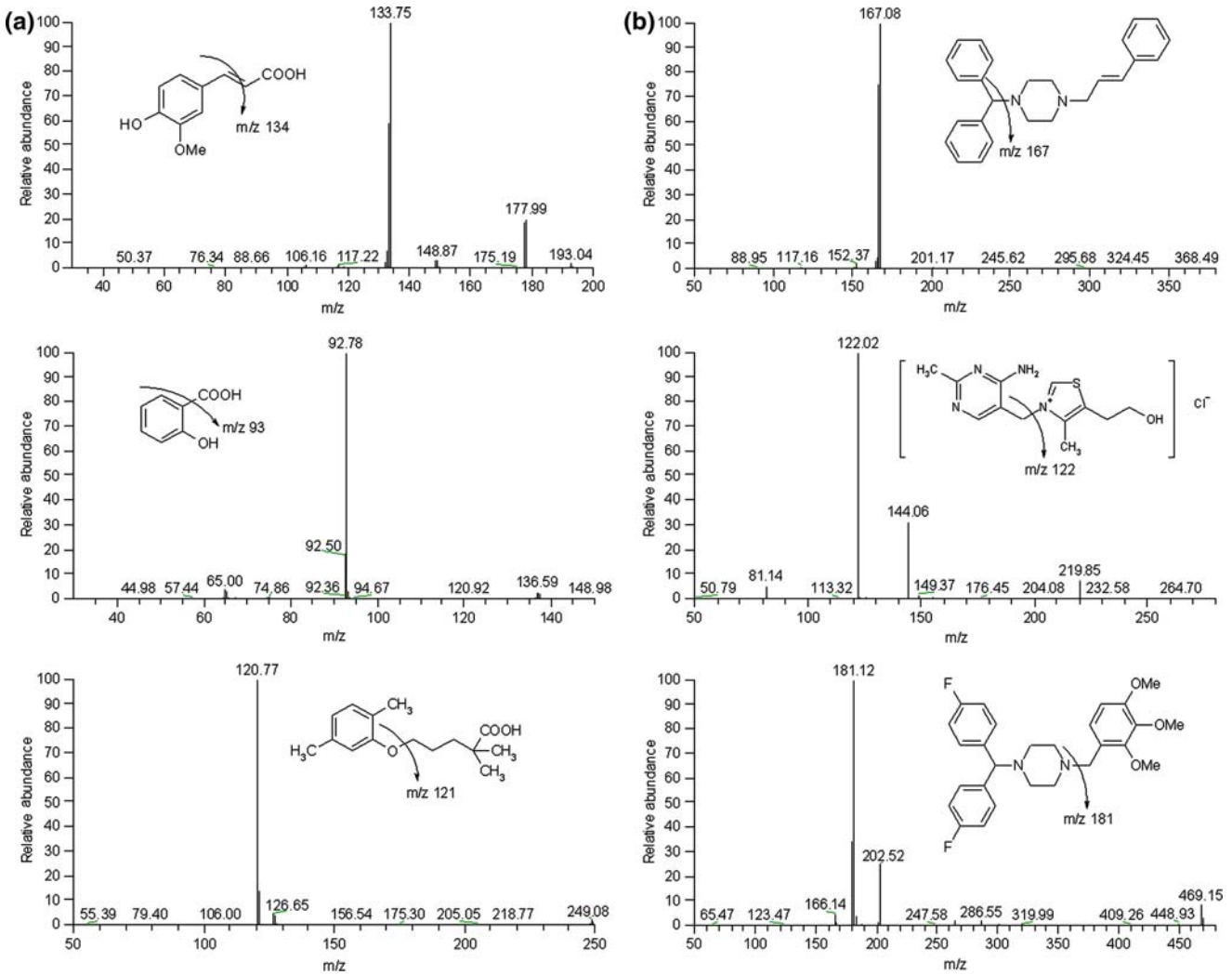

Fig. 1. Full scan ESI (±) production mass spectra and the structures of SF, SA, CIN, VB1 and IS (LOM and GEM)

ciency. It has also been prescribed for motion sickness, and vestibular symptoms of different origins, and gives accurate efficiency for different syndromes such as Meniere's disease or cerebrovascular vertigo [6, 7]. VB1 known as thiamine plays an important role in metabolizing carbohydrates and fat for producing energy. It is essential for normal growth and development and helps maintain proper function of the heart and the nervous and digestive systems. When used in combination (50 mg SF, 20 mg aspirin, 25 mg CIN and 10 mg VB1) the effects were significantly increased for cardiovascular and cerebrovascular diseases with less adverse effects.

It was reported that low-dose aspirin had fewer adverse bleeding events, indicating that adjusting dosages cautiously in certain patients would lower the risks of dosages related side effect [8]. CIN a calcium antagonist suggested that it could produce Parkinsonism in healthy primates in a dose dependent pattern [9]. In addition, the therapeutic daily dose of 50 mg for SF, 20 mg for aspirin, 25 mg for CIN and 10 mg for VB1 given orally resulted in lower plasma concentrations in the elimination phase. Therefore, a sensitive, rapid and simple method for the simultaneous determination of plasma concentrations of SF, salicylic acid (SA), which is the active metabolite of aspirin, CIN and VB1, is required to study their pharmacokinetics or bioavailability.

Various analytical methods have been reported for quantitative determination of SF, SA, CIN and VB1 individually in biological fluids, such as liquid chromatography with ultraviolet detector (HLC–UV) or liquid chromatography–electrospray ionization–mass spectrometry (HLC–ESI–MS) for SF, gas chromatography (GC), capillary electrophoresis (CE) and HLC–UV for SA, HLC–UV, GC or thin-layer chromatography for CIN, HPLC–UV for VB1. However, these methods often suffered from several disadvantages including a complex sample preparation [10–12], low sensitivity or long running

time [13–15], a complex and time-consuming analytical procedure and/or derivatization either pre-column or post-column which increased the complexity of chromatograms [16–20]. Some methods [21, 22], which can satisfy the quantitation of a drug in biological fluids selectively and sensitively, could unfortunately not be applied for simultaneous determination of the four drugs.

To date, no bioanalytical method has been reported in the literature describing the full details of a complete methodology and validation for simultaneously quantifying SF, SA, CIN and VB1 using liquid chromatography with tandem mass spectrometric detection (LC–MS–MS). In the proposed assay, a sensitive, rapid and simple method employing single step protein precipitation for sample extraction and LC–MS–MS for the simultaneous quantification of SF, SA, CIN and VB1 in human plasma and its application to a clinical study was developed.

Experimental

Chemicals and Reagents

Sodium ferulate and aspirin test capsules (batch NO.20060221) were obtained from Horus C&K Pharmaceutical Company (Zhenzhou, P.R. China); SF reference standard (≥98.5% purity), SA reference standard (≥98.5% purity), CIN reference standard (≥98.5% purity) and VB1 reference standard (≥98.5% purity) were all supplied by Dezhou Deyao Pharmaceutical Company (Dezhou, P.R. China); Gemfibrozil (GEM) reference standard (internal standard (IS), 99.7% purity) was supplied by Chengdu Hengrui Pharmaceutical Company (Chengdu, P.R. China). Lomerizine (LOM) reference standard (IS, 99.7% purity) was supplied by C&O Pharmaceutical Technology (Nanjing, P.R. China). Acetic acid and ammonium acetate (analytical reagent) were purchased from Nanjing Chemical Reagent No.1 Factory. HLC grade acetonitrile was purchased from VWR International Company (Darmstadt, Germany). Other chemicals were all of analytical grade. Water was distillated twice before use.

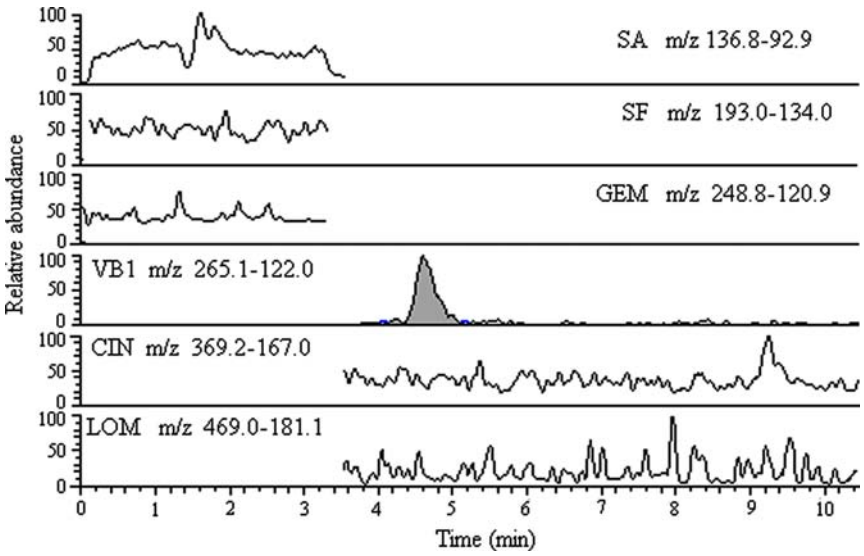

Fig. 2. Representative SRM chromatograms for SF, SA, CIN and VB1 resulting from analysis of blank plasma (drug and IS free)

Instrumentation

LC–MS–MS analysis was performed using a Finnigan TSQ Quantum Discovery MAX system consisting of Finnigan Surveyor LC pump, a Finnigan Surveyor auto sampler, a vacuum degasser, and combined with a triple quadrupole TSQ Quantum mass spectrometer (Thermo Electron Corporation), run by Xcalibur 2.0 software (Thermo Electron Corporation).

Chromatographic Condition

Samples were separated on an Agilent Zorbax SB-CN column (150 mm × 2.0 mm ID, 5 μm) that was maintained at 40 °C. The column was purchased from Aglient Technologies (California, USA). The mobile phase consisted of 65% acetonitrile and 35% (*v*/*v*) aqueous ammonium acetate solution (10 mM, pH was adjusted to 5.0 with acetic acid) at a flow-rate of 0.2 mL min^{-1}.

Mass Spectrometric Condition

Tandem mass spectrometric detection (MS–MS) was operated at unit resolution in the selected reaction monitoring mode (SRM). The electrospray ionization source (ESI) was set at negative ionization mode in 0–3.5 min for SF, SA and GEM, while at positive ionization mode in 3.5–10.5 min for CIN, VB1 and LOM. Transition ions *m*/*z* 193.0→134.0, 136.8→92.9, 369.2→167.0, 265.1→122.0, 248.8→120.9 and 469.0→181.1 for SF, SA, CIN, VB1, GEM and LOM were selected. In the positive and negative modes, source conditions were optimized as follows: spray voltage was at 4,500/−4,000 V, transfer capillary temperature at 290 °C, sheath gas and auxiliary gas (nitrogen) pressure at 30 and 8 arbitrary units (set by the LCQ software, Thermo Electron Corporation). Argon was used as collision gas at a pressure of 1.5 mTorr, meanwhile, collision energy were 17, 18, 23, 15, 18 and 20 eV for SF, SA, CIN, VB1, GEM and LOM, respectively. The scan width for SRM was 0.01 *m*/*z* and scan time was 0.1 s. The peak width settings (FWHM) for both Q1 and Q3 were 0.7 *m*/*z*.

Preparation of Sock and Working Solutions

The stock solutions of SF (1 mg mL^{-1}) were prepared in acetonitrile and stored at 4 °C protected from light until used in order to prevent SF from decomposing.

Table 1. The results of five calibration curves for determining SF in human plasma

Concentration added (ng mL^{-1})	Assay	1.5	4	10	40	100	400	1,000
Concentration found (ng mL^{-1})	1	1.41	4.50	9.56	43.79	109.03	391.10	953.78
	2	1.35	4.30	10.35	37.94	103.84	389.91	985.31
	3	1.54	4.50	10.02	39.45	105.16	393.26	893.53
	4	1.67	3.78	8.64	36.17	89.26	379.34	978.35
	5	1.35	4.11	11.00	41.16	110.54	414.03	929.14
Mean (ng mL^{-1})		1.46	4.24	9.91	39.70	103.57	393.53	948.02
SD (ng mL^{-1})		0.14	0.30	0.89	2.94	8.46	12.66	37.65
Precision (%)		9.73	7.19	8.94	7.40	8.16	3.22	3.97
Accuracy (%)		97.63	105.93	99.14	99.26	103.57	98.38	94.80

Table 2. The results of five calibration curves for determining SA in human plasma

Concentration added (ng mL^{-1})	Assay	20	50	200	500	1,000	2,000	5,000
Concentration found (ng mL^{-1})	1	20.71	52.03	191.22	472.08	1042.33	1971.93	4642.33
	2	22.02	55.44	199.73	497.34	1034.32	1871.87	4247.17
	3	21.61	49.53	210.01	517.27	986.73	2045.94	4574.29
	4	16.48	44.86	196.32	526.68	1135.19	2119.89	4615.645
	5	19.40	46.61	192.12	547.81	1056.37	2158.35	4722.75
Mean (ng mL^{-1})		20.04	49.69	197.88	512.23	1050.99	2033.60	4560.43
SD (ng mL^{-1})		2.23	4.22	7.59	28.87	53.85	115.24	183.32
Precision (%)		11.13	8.50	3.84	5.64	5.12	5.67	4.02
Accuracy (%)		100.22	99.39	98.94	102.45	105.10	101.68	91.21

Table 3. The results of five calibration curves for determining CIN in human plasma

Concentration added (ng mL^{-1})	Assay	2	5	20	50	100	200	500
Concentration found (ng mL^{-1})	1	1.95	5.51	20.72	53.35	103.75	187.67	507.33
	2	1.85	4.89	17.49	50.28	100.11	193.35	498.75
	3	2.02	4.39	21.65	44.59	112.15	179.19	473.58
	4	2.20	4.88	20.48	53.79	103.53	176.15	498.91
	5	1.97	5.24	21.24	52.09	104.53	190.76	492.01
Mean (ng mL^{-1})		2.00	4.98	20.32	50.82	104.81	185.43	494.12
SD (ng mL^{-1})		0.13	0.42	1.64	3.74	4.44	7.43	12.70
Precision (%)		6.36	8.45	8.08	7.36	4.24	4.01	2.57
Accuracy (%)		99.89	99.63	101.58	101.64	104.81	92.71	98.82

Table 4. The results of five calibration curves for determining VB1 in human plasma

Concentration added (ng mL^{-1})	Assay	1	2	5	10	15	20	30
Concentration found (ng mL^{-1})	1	1.06	1.71	4.47	8.80	14.31	21.58	32.97
	2	1.07	1.76	4.73	9.35	13.55	22.31	33.43
	3	1.07	1.82	4.46	9.20	13.24	21.84	32.14
	4	1.04	1.89	4.73	9.02	13.37	22.56	34.31
	5	1.10	1.78	4.96	9.73	13.26	22.02	34.11
Mean (ng mL^{-1})		1.07	1.79	4.67	9.22	13.55	22.06	33.39
SD (ng mL^{-1})		0.02	0.07	0.21	0.35	0.44	0.38	0.88
Precision (%)		1.96	3.64	4.50	3.83	3.26	1.74	2.64
Accuracy (%)		107.03	89.67	93.38	92.19	90.31	110.31	111.30

The stock solutions of SA (1 mg mL^{-1}), CIN (1 mg mL^{-1}), GEM (1 mg mL^{-1}) and LOM (1 mg mL^{-1}) were all prepared in acetonitrile and stored at 4 °C. The stock solutions of VB1 (1 mg mL^{-1}) were prepared in water and stored at 4 °C. Working solutions of SF, SA, CIN, VB1 were prepared daily by appropriate dilution of their stock solutions in acetonitrile–water (65:35, *v*/*v*) in the required concentration range. The IS working solution, containing 1 μg mL^{-1} of GEM and 1 μg mL^{-1} of LOM, was prepared by diluting the stock solution of GEM and LOM with diluent. All the solutions were stored at 4 °C.

Sample Preparation

To 200 μL plasma in a 1.5 mL polypropylene micro-centrifuge tube, 10 μL of the IS working solution (1 μg mL^{-1} of GEM and LOM) and 0.4 mL acetonitrile were added. The mixture was vortex-mixed thoroughly for 3 min and then centrifuged at 13,772 *g* for 5 min. The supernatant was transferred to another clean tube and centrifuged again at 13,772 *g* for 8 min. Then an aliquot of 10 μL supernatant was directly injected into the LC–MS–MS system. The sample preparation must be protected from light in order to prevent SF from decomposing.

Bioanalytical Method Validation

The method validation assays were carried out according to the United States Food and Drug Administration (FDA) bioanalytical method validation guidance (Food and Drug Administration, 2001).

Calibration curves were generated by using the ratios of the analyte peak area to the IS peak area versus concentration and were fitted to the equation $y = bx \pm a$ by weighted least-squares linearity regression ($1/\chi^2$). Blank plasma samples of healthy human used for testing specificity of the method were obtained from six different sources. Calibration curves were prepared on five different days by spiking blank plasma

with proper volume of one of the working solutions mentioned above to produce the standard curve points equivalent to 1.5, 4, 10, 40, 100, 400 and 1,000 ng mL^{-1} of SF, 20, 50, 200, 500, 1,000, 2,000 and 5,000 ng mL^{-1} of SA, 2, 5, 20, 50, 100, 200 and 500 ng mL^{-1} of CIN, 1, 2, 5, 10, 15, 20 and 30 ng mL^{-1} of VB1. The following assay procedures were the same as that described above. Blank plasma samples (without IS) were also analyzed.

Quality control (QC) samples were prepared by spiking blank plasma with proper volume of one of the working solutions mentioned above to produce a final concentration equivalent to 4, 100, 1,000 ng mL^{-1} of SF, 20, 500, 5,000 ng mL^{-1} of SA, 2, 50, 500 ng mL^{-1} of CIN, 1, 10, 30 ng mL^{-1} of VB1. The following procedures were the same as that described above.

The matrix effect (ME) was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample. It was examined by comparing the peak areas of the analytes and IS between the different sets of samples. In set 1, analytes were dissolved in the reconstituted solution of blank plasma sample, and the obtained peak areas of analytes were defined as *A*. In set 2, analytes were dissolved in mobile phase, and the obtained peak areas of analytes were defined as *B*. In set 3, the obtained peak areas of VB1 in the blank plasma sample were defined as *C*. ME for SF, SA, CIN, IS were calculated by using the formula: ME (%) = $A/B \times 100$. The matrix effect of the method was evaluated at three concentration levels of 4,100,1,000 ng mL^{-1} for SF, 20,500,5,000 ng mL^{-1} for SA, 2, 50, 500 ng mL^{-1} for CIN. The matrix effect of GEM and LOM (IS, 1 μg mL^{-1} in plasma) was evaluated using the same method. ME for VB1 was calculated by using the formula: ME (%) = $(A - C)/B \times 100$. The matrix effect of the method was evaluated at three concentration levels of 1, 10, 30 ng mL^{-1} for VB1. Five samples at each concentration level of the analytes were analyzed. The blank plasma samples used in this study were collected from five different batches of human blank plasma. If the ME values exceed the range of 85–115%, an exogenous matrix effect is implied.

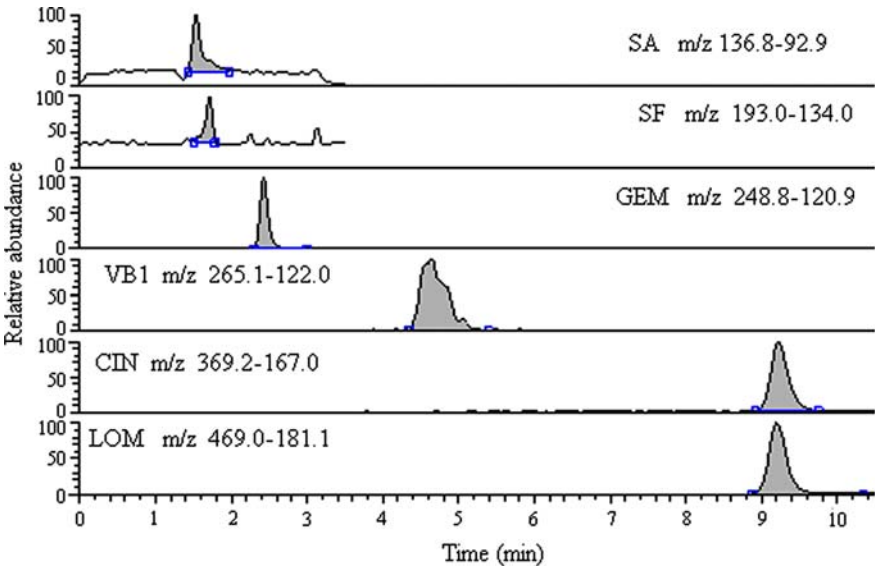

Fig. 3. Representative SRM chromatograms for SF, SA, CIN and VB1 resulting from analysis of the LLOQ

The intra-batch precision and accuracy were determined by analyzing five sets of spiked plasma samples of the analytes at each QC level in a day. The inter-batch precision and accuracy were determined by analyzing five sets of spiked plasma samples of the analytes at each QC level in three consecutive days. The concentration of each sample was calculated using standard curve prepared and analyzed on the same day.

The relative extraction recoveries (RE) were assessed by comparing the analytes to the IS peak area ratios obtained from extracted samples spiked with a known amount of the analytes with those from post-extraction blank plasma samples spiked at corresponding concentrations. In set 1, the analytes to the IS peak area ratios obtained from extracted samples spiked with a known amount of the analytes were defined as *As-r*. In set 2, the ratios from post-extraction blank plasma samples spiked at corresponding concentrations were defined as *Ar-r*. In set 3, the VB1 to the LOM peak area ratios from the blank plasma sample were defined as *Ao-r*. RE for SF, SA, CIN was calculated by using the formula: RE (%) = $As\text{-}r/Ar\text{-}r \times 100$, while for VB1, RE (%) = $(As\text{-}r - Ao\text{-}r)/(Ar\text{-}r - Ao\text{-}r) \times 100$. This procedure was repeated ($n = 5$) at each QC concentration level for each analytes.

The auto-sampler stability was measured by determining QC samples kept under the auto-sampler condition (15 °C) for 24 h. The freeze thawing stability was tested by analyzing QC plasma samples under freeze condition (−20 °C) for 10 days. Three spiked plasma samples at each QC level were analyzed for the stability studies.

Results and Discussion

LC–MS–MS Optimization

In this study, ESI was selected as the ionization source. And the mass spectrometer was tuned in both positive and negative ionization modes to check for optimum response of SF, SA, CIN, VB1 and the IS. It was found that CIN and VB1 exhibited poor sensitivity in the negative-ion mode, while SA and SF exhibited poor sensitivity in the positive-ion mode. Accordingly, it was decided to detect SA, SF and GEM (IS) in the negative mode, followed by the detection of CIN, VB1 and LOM (IS) after switching to the positive mode.

The MS–MS conditions were optimized and the full product spectra of all

Table 5. The precision and accuracy of the method for determining SF, SA, CIN, VB1 in human plasma

Concentration added (ng mL^{-1})		Inter-batch (n = 15)			Intra-batch (n = 5)		
		Concentration found (mean ± SD, ng mL^{-1})	Accuracy (%)	Precision (%)	Concentration found (mean ± SD, ng mL^{-1})	Accuracy (%)	Precision (%)
SF	4	4.10 ± 0.41	102.41	9.90	4.03 ± 0.45	100.71	11.22
	100	99.06 ± 6.99	99.06	7.06	97.44 ± 7.32	97.44	7.51
	1,000	967.17 ± 37.29	96.72	3.86	945.28 ± 26.13	94.53	2.76
SA	20	20.49 ± 2.16	102.45	10.53	21.09 ± 1.91	105.47	9.08
	500	484.31 ± 31.32	96.86	6.47	498.31 ± 31.67	99.66	6.36
	5,000	5007.59 ± 145.45	100.15	2.90	4995.81 ± 133.04	99.42	2.66
CIN	2	2.01 ± 0.23	100.32	14.10	2.04 ± 0.22	101.89	10.69
	50	47.16 ± 3.32	94.33	6.64	50.24 ± 2.84	100.49	5.65
	500	496.77 ± 17.68	99.35	3.41	499.93 ± 18.31	99.99	3.66
VB1	1	1.01 ± 0.11	100.5	10.60	0.96 ± 0.12	95.59	12.51
	10	10.40 ± 0.65	103.97	6.22	10.7 ± 0.64	107.35	5.99
	30	30.20 ± 1.27	100.66	4.22	31.00 ± 1.24	103.33	4.00

compounds were acquired. According to the full scan (-) mass spectra, the ion $[M\text{-}Na]^-$ m/z 193.0 for SF, $[M\text{-}H]^-$ m/z 136.8 for SA and $[M\text{-}H]^-$ m/z 248.8 for GEM were selected as the precursor ion to obtain the product ion. The most sensitive ion transitions were m/z 193.0→134.0 for SF, m/z 136.8→92.9 for SA and m/z 248.8→120.9 for GEM, respectively. Full scan ESI (−) product ion mass spectra and their structure are shown in Fig. 1a. According to the full scan (+) mass spectra, the ion $[M+H]^+$ m/z 369.2 for CIN, $[M\text{-}Cl]^+$ m/z 265.1 for vitaminB1 and $[M+H]^+$ m/z 469.0 for LOM were selected as the precursor ion to obtain the product ion. The most sensitive ion transitions were m/z 369.2→167.0 for CIN, m/z 265.1→122.0 for VB1 and m/z 469→181.1 for LOM, respectively. Full scan ESI (+) product ion mass spectra and their structure are shown in Fig. 1b.

Therefore, transition ions m/z 193.0→134.0 for SF, m/z 136.8→92.9 for SA and m/z 248.8→120.9 for GEM (IS) in the negative mode were selected for monitoring in 0–3.5 min. Then transition ions m/z 369.2→167.0 for CIN, m/z 265.1→122.0 for VB1 and m/z 469.0→181.1 for LOM (IS) in the positive mode were selected for monitoring in 3.5–10.5 min.

Considering the different polarity of the analytes, VB1 tended to have different retention pattern than others. Using reverse-phase columns, such as C18 column, SF, SA and CIN had a suitable retention time while VB1 had a poor retention property. Thus, the Agilent Zorbax SB-CN column, was adopted because for its bonded-phases were more polar than common C8 and C18 columns. The retention for non-polar compounds may be reduced substantially while the retention is maintained for polar compounds.

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials including investigating various ammonium acetate buffer concentrations and pH values to obtain the suitable retention time of the analytes for simultaneous determination. It was found that the peak intensity decreased with the increase in the concentration of ammonium acetate buffer, while the retention time of CIN and VB1 obviously decreased. When lowering the pH values, the retention time of VB1 decreased while CIN increased. Based on these results, the adopted mobile phase, a mixture of acetonitrile and 10 mM ammonium acetate buffer (65:35, v/v, pH adjusted to 5.0 with acetic acid) well balancing the chromatographic separation and MS-MS sensitivity, has successfully separated SF, SA, CIN and VB1, as well as the IS.

Sample Preparation

VB1 had a very high polarity, so it was impossible to extract it from biological fluids using liquid–liquid extraction. In the end, a protein precipitation method was selected. To obtain high extraction efficiency, three different protein precipitation agents, acetonitrile, methanol and 20% perchloric acid, were investigated. It was found that acetonitrile did not significantly affect the mass response compared to the other protein precipitation agents. Finally, a simple single-step protein precipitation with acetonitrile was adopted.

The Determination of VB1

Considering VB1 was an endogenous drug, calibration curves were constructed by determining the best fit of peak area ratios (the difference between the peak area of VB1 in the spiked plasma and in the blank plasma/peak area of LOM) versus concentration. Their relationship fitted to the equation $y = bx \pm a$ by weighted linear regression ($1/\chi^2$), where y corresponded to the peak area ratio and x referred to the concentration of VB1 which was added to the plasma. The QC concentration levels of VB1 differed between the concentration of VB1 in the spiked plasma and in the blank plasma.

Assay Selectivity and Matrix Effect

No analyte-interfering peaks were observed due to the high selectivity of SRM. Drug-free plasma was precipitated with acetonitrile and the results were recorded. Figure 2 showed the representative HLC chromatograms for a drug free plasma

sample, indicating that no endogenous peaks were present at the retention time (t_R) of the analytes or of the IS except for VB1. And all the ratios of the peak area of the analytes dissolved in the supernatant of the processed blank plasmas compared to that of standard solutions at the same concentration were between 85 and 115%. The results showed that there was no significant difference in peak areas. It indicated that no co-eluting 'invisible' compounds significantly influenced the ionization of the derivatives of analytes and IS.

Linearity of Calibration Curves and Specificity

Assay linearity was evaluated by calibration curves ranging from 1.5 to 1,000 ng mL^{-1} for SF, 20–5,000 ng mL^{-1} for SA, 2–500 ng mL^{-1} for CIN and 1–30 ng mL^{-1} for VB1. Using linear regression analysis, an excellent linear relationship between peak area ratio and concentrations was exhibited for SF $R = 0.001522C–0.0009488$, $r = 0.9991$, for SA $R = 0.003432C–0.007070$, $r = 0.9988$, for CIN $R = 0.1405C–0.1205$, $r = 0.9992$ and for VB1 $R = 0.03045C–0.02471$, $r = 0.9928$. The use of the weighted regression resulted in less than 15% deviation between the nominal and experimental concentrations calculated from the equations. Results of five representative standard curves for LC–MS–MS determination of each analyte were given in Tables 1, 2, 3 and 4.

The lower limit of quantification (LLOQ) is defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision. Figure 3 depicted representative HLC chromatograms for the LLOQ, 1.5 ng mL^{-1} for SF, 20 ng mL^{-1} for SA, 2 ng mL^{-1} for CIN, 1 ng mL^{-1} for VB1, respectively.

Precision and Accuracy

Data for intra- and inter-batch precision and accuracy of the method for SF, SA, CIN and VB1 were presented in Table 5. The precision deviation values for intra-batch and inter-batch were all within 15% of the relative standard deviation (RSD) at each QC level. The accuracy deviation values for intra- and inter-batch were all within 100 ± 15% of the actual values at each QC level. The results revealed good precision and accuracy.

Table 6. The RE of SF, SA, CIN and VB1 in human plasma

Concentration added (ng mL^{-1})		RE (%)					Mean	RSD (%)
SF	4	94.68	87.8	83.42	80.83	99.24	89.19	8.61
	100	87.98	89.46	86.18	84.04	81.59	85.85	3.64
	1,000	85.74	83.97	90.88	88.87	88.83	87.66	3.15
SA	20	79.15	85.87	71.57	93.2	75.98	81.15	10.49
	500	72.36	81.76	77.85	79.66	83.74	79.07	5.51
	5,000	78.53	85.31	84.48	82.45	79.58	82.07	3.61
CIN	2	110.95	98.4	96.42	82.10	98.74	97.32	10.55
	50	87.58	94.91	103.83	94.50	102.08	96.58	6.77
	500	93.52	100.33	98.15	100.67	100.90	98.72	3.14
VB1	1	78.32	84.19	94.61	72.40	91.21	84.15	10.81
	10	79.81	78.95	83.45	89.12	76.47	81.56	6.02
	30	86.14	85.89	86.59	86.04	82.31	85.4	2.04

Table 7. The stability of SF, SA, CIN and VB1 in human plasma under tested conditions

	Accuracy (mean %)					
	SF			SA		
Concentration added (ng mL^{-1})	4	100	1,000	20	500	5,000
Auto sampler stability (48 h, 15 °C)	96.02	95.17	96.13	88.12	98.97	102.47
Freeze thawing stability(10 days, −20 °C)	99.95	99.02	97.87	96.47	100.81	91.56

	CIN			VB1		
Concentration added (ng mL^{-1})	2	50	500	1	10	30
Auto sampler stability (48 h, 15 °C)	116.67	94.10	99.19	94.58	106.46	96.35
Freeze thawing stability(10 days, −20 °C)	107.12	98.94	101.27	96.79	98.07	103.20

Extraction Efficiency

The data of extraction efficiency measured for the analytes and the IS in human plasma were consistent, precise and reproducible. The result of the mean relative extraction recoveries and the RSD of the analytes is seven in is given in Table 6.

Analyte Stability

Table 7 summarizes the results of the stability of SF, SA, CIN and VB1 through freeze thawing and in auto sampler. The data showes the reliable stability behavior of SF, SA, CIN and VB1 under the tested condition.

Application of the Analytical Method to a Pharmacokinetic Study

The validated method was successfully used to quantify SF, SA, CIN and VB1 concentrations in a preliminary pharmacokinetic study. After fasted overnight, eight volunteers were administrated sodium ferulate and aspirin test capsules (containing 50 mg SF, 20 mg aspirin, 25 mg CIN and 10 mg VB1) in the single dose study. Then serial blood samples were collected from the vein at 0.17, 0.25, 0.33, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12, 24 and 48 h post-dose. They were transferred into lithium heparin tubes and were immediately centrifuged at 2,000 *g* for 10 min. The plasma obtained was frozen at −20 °C in coded

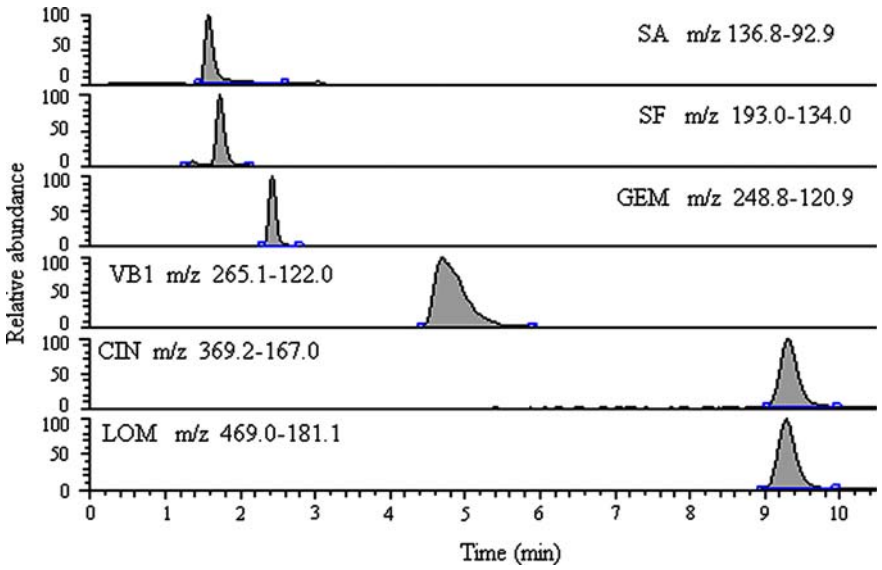

Fig. 4. Representative SRM chromatograms of a plasma sample obtained at 0.33 h from a subject who received a single oral dose of ferulate and aspirin test capsules compound sodium

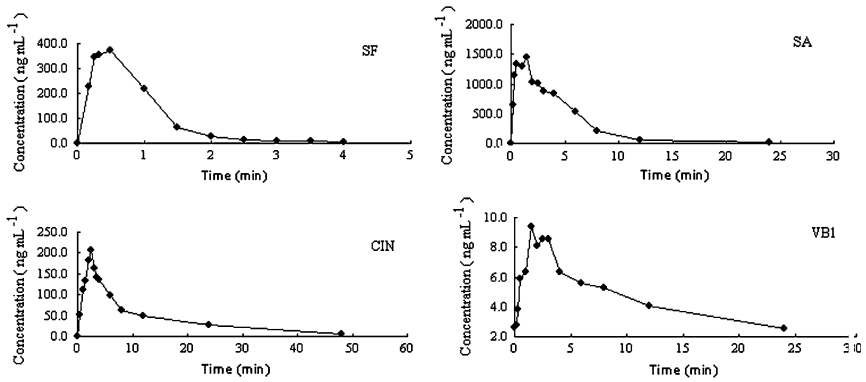

Fig. 5. Mean drug plasma concentration time curve of SF, SA, CIN and VB1 from the eight subjects after oral administration

polypropylene tubes until analysis. A representative chromatogram of a plasma sample obtained at 0.33 h from a subject who received a single oral dose of compound sodium ferulate and aspirin test capsules is shown in Fig. 4. The pharmacokinetic profiles of SF, SA, CIN and VB1 from the eight subjects after oral administration are shown in Fig. 5.

Conclusions

The paper presents a rapid, selective and sensitive LC–MS–MS method for simultaneous determination of SF, SA, CIN and VB1 in human plasma with a total running time of 10.5 min for each injection. Considering the different properties of SF, SA, CIN and VB1, it was decided to extract these drugs simultaneously with a single protein precipitation step and detect SF, SA in the negative mode, followed by the detection of CIN and VB1 after switching to the positive mode. The method was successfully applied to quantify the concentration–time profiles of SF, SA, CIN and VB1 simultaneously in a clinical pharmacokinetic study.

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