Tropical Journal of Pharmaceutical Research March 2020; 19 (3): 651-659
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i3.28

Original Research Article

Comparative pharmacokinetic study of five flavonoids in normal rats and rats with gastric ulcer following oral administration of Mongolian medicine, Shudage - 4 by UPLC – ESI – MS/MS

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Sent for review: 29 September 2019 *Revised accepted: 23 February 2020*

Abstract

Purpose: *To develop a simple, rapid and sensitive ultra-performance liquid chromatography - electrospray ionization-mass spectrometry (UPLC–ESI–MS/MS) method was developed and fully validated for the simultaneous determination of galangin, kaempferide, galangin-3-methylether, kaempferol and quercetin in rat plasma after oral administration of Mongolian Medicine, Shudage-4 extracts.*

Methods: *The galangin, kaempferide, galangin-3-methylether, kaempferol and quercetin were separated on a C_{18} column using 0.1 % formic acid at a flow rate of 0.4 mL / min and detected by a mass spectrometer in negative-ion mode with selected reaction monitoring (SRM) mode. Plasma samples were processed with a simple deproteinization technique using ethyl acetate and acetonitrile. Following the protein precipitation, the plasma samples were evaporated under gentle stream of nitrogen and analyzed by above method. Naringin was used as an internal standard (IS). Method validation was performed according to the Chinese Food and Drug Administration guidelines.*

Results: *A good linearity ($r^2 \geq 0.9990$) was showed by the UPLC – ESI – MS / MS method, the low limits of quantification for galangin, kaempferide, galangin-3-methylether, kaempferol and quercetin were 229.8, 78.8, 32.0, 123.7 and 137.8 ng / mL, respectively. The results of inter-day and intra-day precisions met the experimental requirement (< 7.8 %). The matrix effect and recovery efficiency of the five analytes were more than 72.9 and 88.7 % respectively. The stability of the analytes were satisfactory. The UPLC – ESI – MS / MS method has been used for the five analytes' pharmacokinetics study successfully after gastrointestinal route of the Mongolian Medicine Shudage-4. The pharmacokinetic parameters showed significant differences ($P < 0.05$) between the normal and gastric ulcer groups. The metabolism and transport of the five analytes in gastric ulcer rates were faster than in normal rats after administration of Shudage - 4 extract. Double-peak phenomenon appeared in galangin, galangin – 3 - methylether and quercetin.*

Conclusion: *The results suggest that the metabolism and transport of Mongolian Medicine Shudage-4 in gastric ulcer rats is faster than in normal rats and may be enriched and acted on at the lesion site.*

Keywords: *UPLC – ESI – MS / MS; Mongolian medicine; Shudage - 4; pharmacokinetics; gastric ulcer*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

On account of the cold climate, meal-rich and wine-rich diet in Mongolia area, stomach diseases occur frequently. Over the years, the Mongolian people have accumulated rich experience in treatment of stomach diseases, and some unique Mongolian medicines have shown good curative effects in the treatment of stomach diseases.

Shudage - 4, which consists of galangal, purpurea halitium, costustoot and acorus gramineus soland, is a common prescription for stomach disorder. It has been widely used in clinical settings for treating stomach illness for hundreds of years in Mongolia [1]. Phytochemical studies have revealed that flavonoids, including *galangin, kaempferide, galangin-3-methylether, kaempferol* and *quercetin*, are major ingredients in *Shudage-4* [2,3-5]. Their quality control and pharmacological efficacy have been reported [6-8]. The pharmacological activity of flavonoids has strong antioxidative action, free radical scavenging capacity, hepatoprotective effect, antibacterial action, anti-inflammatory, and anticancer activity [9]. In addition, antiviral activity has also been recognized since the 1940s, such as anti - human immunodeficiency virus (anti-HIV), anti - herpes simplex virus (anti - HSV), and anti - dengue virus [10].

Several analytical methods based on high performance liquid chromatography (HPLC), ultraviolet spectrophotometry (UV), liquid chromatograph-mass spectrometer (LC-MS) have been reportedly used to evaluate the main flavonoids in rat plasma. In order to better separation in plasma samples, HPLC and UV methods not only required a long chromatographic process to avoid the interference, but also the unsatisfactory sensitivity for determinations was the most important problem. Furthermore, not all flavonoids have active chromophores at appropriate wavelengths or fluorescent agents [11]. Ultra-performance liquid chromatography-electrospray ionization-mass spectrometry (UPLC–ESI–MS/MS) method is currently attracting considerable interest as a simple and sensitive method in qualitative and quantitative analysis, which can overcome most of the above-mentioned limitations [12].

Pharmacokinetic studies of Mongolian medicines play an important part in evaluating clinical efficacy and guiding rational drug usage. Pharmacokinetic studies play an important role in evaluating the rationally of Mongolian medicine prescriptions. No reported on the pharmacokinetic of *Shudage - 4* extract and no simultaneous determination of *galangin, kaempferide, galangin-3-methylether, kaempferol* and *quercetin* by the UPLC – ESI – MS/MS method. In consequence, the pharmacokinetics of the above analytes in gastric ulcer model and normal rat plasma were compared.

To exert a biological effect, the herbs of prescriptions must act on the target tissues after oral administration. The pharmacokinetic studies are responsible for determine concentration of the main bioactive contents in vivo, elucidate efficacy, explain drug action, and optimize dosage.

Therefore, to further investigate the pharmacokinetics and interaction, a rapid, accurate and sensitive UPLC–ESI–MS/MS method was developed for the simultaneous determination of *galangin, kaempferide, galangin–3-methylether, kaempferol* and *quercetin* in rat plasma after oral administration of *Shudage - 4* extracts using naringin as internal standards.

EXPERIMENTAL

Chemicals and reagents

Galangin–3-methylether (NO.20170919001), kaempferide (NO.20160328), kaempferol (NO. MUST - 17040502) and *quercetin (NO.100081-200406)* and internal standard *(IS) Naringin (NO.110722 - 201714)* were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). *Galangin* was previously isolated and identified by MS and NMR spectra. Their purity was all above 98% analyzed by HPLC. HPLC-grade methanol and formic acid were purchased from Thermo Fisher Scientific (CN, USA).

Instrumentation and chromatographic conditions

Samples were analyzed by a TSQ quantum ultra-mass spectrometric detector with electrospray ionization source (ESI) (Termo Scientific, USA) coupled with a Dionex Ultimate 3000 ultra - performance liquid chromatography system. The operating conditions for mass were as follows: 3000 V for spray voltage, 300 ℃ for vaporizer temperature, 35 AU for sheath gas (Arb) pressure; 10 AU for auxiliary gas (Arb) pressure; 5 AU for ion sweep gas (Arb) pressure, and 300 ℃ for capillary temperature. All the operations were under the condition of Xcalibur software (Thermo Scientific, USA).

Preparation of total flavonoids of *Shudage - 4*

The *Shudage - 4* composed of the dry root of *Alpinia officinarum Hance (400 g), Aucklandia lappa Decne (100 g), Acorus tatarinowii Schott (100 g)* and *Halite Violaceous (200 g)* were extracted and refluxed for 2 hours with 70 % ethanol (1:10, w/v). The extracting solution was combined, and concentrated by reduced the pressure. The concentrated eluent was loaded on macroporous resin column (D101 type), and elution with H_2O and 50 % aqueous ethanol in turn. Collecting the 50 % aqueous ethanol elution and obtaining the flavonoid fraction of *Shudage - 4* [8,13].

Standards' and quality control samples' preparation

The standard stock solution of the five analytes were prepared with the final concentration of 229.8 μg/mL for *galangin*, 3200.0 μg/mL for *galangin–3-methylether*, 788.0 μg/mL for *kaempferide*, 123.7 μg/mL for *kaempferol*, 137.8 μg/mL for *quercetin,* respectively.

The stock solutions and spiking blank plasma were mixed to produce a series of working solutions, which were 229.8-22984.0 ng/mL for *galangin*; 32.0-3200.0 ng/mL for *galangin – 3 - methylether*; 78.8-7880.0 ng/mL for *kaempferide*; 123.7-12366.0 ng/mL for *kaempferol*; 137.8-13780.0 ng/mL for *quercetin*. The quality control samples were produced at concentrations of 574.6, 2298.4, and 22984.0 ng / mL for *galangin*, 80.0, 320.0, and 3200.0 ng/ml for *galangin-3-methylether*, 344.5, 1378.0, and 13780.0 ng/mL for *kaempferide*, 3019.5, 12366.0, and 123660.0 ng/mLfor *kaempferol*, and 344.5, 1378.0, and 13780.0 ng/mLfor *quercetin*. The standard stock solutions and the working solutions were stored at 4 ℃, and the quality control samples were stored at – 20 ℃ [14].

Induction of model rats

All animal protocols were approved by the Committee of Inner Mongolia medical university on Ethics of Animal Experiments (approval no. YKD20190331). Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). Wistar rats were housed in environmentally controlled quarters (about 23 ~ 25 ℃) under a light-dark cycle (12 h: 12 h) for seven days before experiment. Twelve Wistar rats (7 ~ 8 weeks old, weighing 280 ~ 300g) were split into two, half the rats were put into model group, and another half in normal group. The model group rats were given a 56 % Chinese spirits 3 ml/day for three days by intragastric administration and the normal group rats were given physiological saline.

Plasma sample preparation

Frozen plasma was thawed at room temperature, and 400 μL rat plasma in a 10 mL of polypropylene tube was mixed with 20 μL hydrochloric acid (2 mol / mL) and vortexed for 30 s. Then the mixture was added into 100 μL of IS solution (0.986 mg / mL) and vortexed for 60 s. After being placed in a water bath at 80 ℃ for 30 min and the addition of ethyl acetate, the mixed solution was vortexed for 60 seconds and centrifuged for 10 minutes at 3000 × g. The supernatants were transferred into other polypropylene tubes, and the residues were centrifuged at 3000 × g for 10 min after adding acetonitrile and vortexing for 60 s. The two supernatants were merged firstly and then filtrated by microfiltration membrane, and finally dried under a nitrogen stream. Being dissolved in 500 μL of methanol the residues were vortexed and 20 μL solutions were analyzed in the UPLC–ESI–MS/MS system [15-17]. The samples were immediately centrifuged at 3000 × g for 10 min and stored at – 20 ºC until analysis.

Method validation

Based on the China Food and Drug Administration guidelines, the method was validated with respect to specificity, linearity and sensitivity, precision and accuracy, stability, and recovery.

Specificity

The specificity was performed by comparing the selected reaction monitoring (SRM) chromatograms of blank plasma and spiked plasma samples (including *galangin, galangin–3-methylether, kaempferide, kaempferol, quercetin* and naringin (IS).

Linearity and sensitivity

Seven non-zero points of standard plasma samples were assessed in duplicate and analyzed in non - stop for 3 days for preparation of calibration curves. A weighted linear-squares regression model was used to establish the peak area ratio of the concentration of analytes versus standard plasma samples. The sensitivity was assessed by the lowest concentration on the calibration curve with a signal – to - noise (S / N) ≥ 10 by analyzing six replicates.

Precision and accuracy

Intra- and inter-day precision and accuracy of the analyzed samples were selected at three levels (high, medium, and low) in five replicates on 3 non - stop days. The precision and the accuracy were determined by the relative standard deviation (RSD) and the percent relative error (RE %) respectively, and within ± 15 % for the analyzed samples.

Stability

The stability of the five analytes in rat plasma was subjected to three different circumstances (storage for 12 h at ambient temperature, store at – 20 ℃ for three days, three cycles for freeze / thaw) at three different concentrations (high, medium, and low). All the samples were freshly prepared and the percentage deviation within ± 15 % were considered stable.

Extraction recovery and matrix effects

Matrix effects was assessed by comparing the peak areas of standard solutions spiked after extraction with those of the corresponding solution mixture (including galangin, galangin –3-methylether, kaempferide, kaempferol, quercetin and IS) at three replicates concentrations (low, medium and high) in the same level. By comparing the peak areas of extracted samples (low, medium and high concentrations) with that of the standard solutions at the same concentrations with three replicates, the analytes' extraction recovery were assessed.

Pharmacokinetic study

The pharmacokinetic study of *galangin, kaempferide, kaempferol, galangin-3-methylether* and *quercetin* in rat plasma after intragastric administration of *Shudage - 4* was successfully applied. Twelve wistar rats, six normal rats and six model rats, were fasted under normal conditions and given *Shudage - 4* (0.236 g / mL) by oral administration. 400 µL of blood samples were obtained from the rat retro orbital plexus and injected into heparinized tubes at 10, 30, 60, 90, 120, 150, 180, 240, 300, 420 and 480 min.

Statistical analysis

DAS 2.1 software package was used to analyze the concentration-time data of the five analytes for the determination of the pharmacokinetic parameters. Differences in the parameters were evaluated by unpaired *Student's t - test* with the aid of SPSS 20.0 software.

RESULTS

Optimized LC–MS/MS for quantitative analysis

The selected reaction monitoring (SRM) mode was selected for detection in the negative-ion mode. The optimized mass spectrometric parameters and mass spectra for all the analytes are shown in Figure 1, Figure 2 and Figure 3. The gradient elution system was used to chromatographic separation, which composed of 0.1 % (v/v) formic acid water solution (A) and methanol (B) containing 0.1 % (v/v) formic acid at a flow rate of 1 mL/min. The gradient program follows: 0 - 3 min, 10 % B – 45 % B; 3 - 6 min, 45 % B – 65 % B; 6 - 19 min, 65 % B – 85 % B; 19 - 25 min, 85 % B – 100 % B.

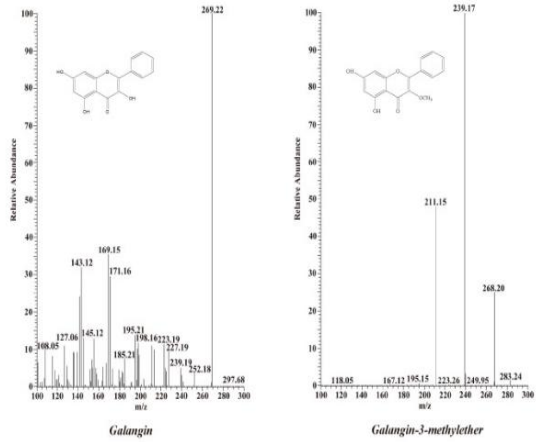

Figure 1: Structure and product ions scan spectra of *galangin* and *galangin – 3 – methylether*

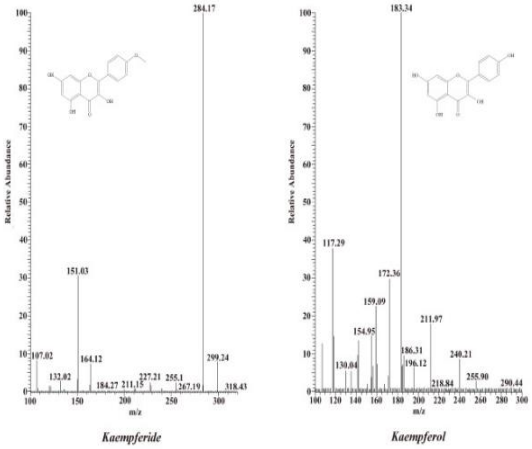

Figure 2: Structure and product ions scan spectra of *kaempferide* and *kaempferol*

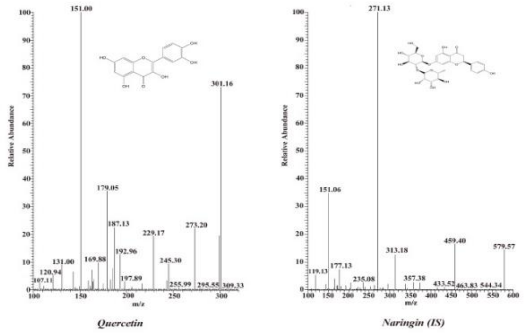

Figure 3: Structure and product ions scan spectra of *quercetin* and *naringin (IS)*

Method validation results

Specificity

As shown in Figure 4, Figure 5 and Figure 6, the specificity was assessed by comparing the chromatograms of blank plasma, spiked plasma samples and IS with good separation. The retention times were 14.46, 15.02, 14.14, 10.49 and 9.36 min respectively for the five analytes. IS was 7.38 min. There was no interference between the five compounds and IS.

Linearity and sensitivity

The linear equations of *galangin, galangin-3-methylether, kaempferidel, kaempferol, quercetin* and their low limits of quantification (LLOQs) were listed in Table 1. The correlation coefficients (r^2) were all above 0.99 for the five compounds and represents good linearity for the five analytes.

Precision and accuracy

The *intra* and *inter* day precision of the five compounds were shown in Table 2, and RSD % were all less than 15 %. For the simultaneous quantitative analysis of the five analytes in rat plasma, the present method was feasible.

Stability

The stability of the five compounds were assessed under different conditions, including storage for 12 h at ambient temperature, store at -20 ℃ for 3 days, and three cycles for freeze/thaw. The UPLC - ESI – MS / MS method was satisfied in stability for the simultaneous determination for the five analytes in rat plasma.

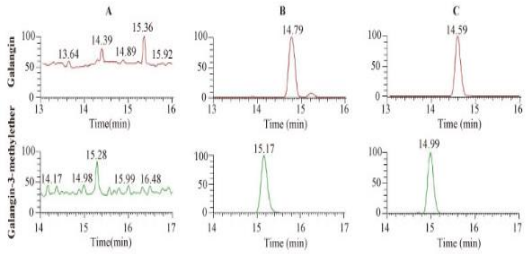

Figure 4: Typical chromatograms of *galangin* and *galangin – 3 - methylether* in rat plasma: (A) blank plasma; (B) blank plasma mixed with *galangin* and *galangin – 3 - methylether*; (C) plasma samples collected from rats after 1.0 h after being given *galangin* and *galangin – 3 – methylether*

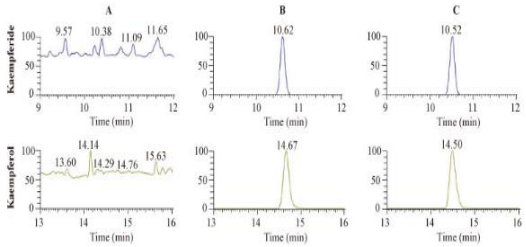

Figure 5: Typical chromatograms of *kaempferide* and *kaempferol* in rat plasma: (A) blank plasma; (B) blank plasma mixed with *kaempferide* and *kaempferol*; (C) plasma samples collected from rats after 1.0 h after being given *kaempferide* and *kaempferol*

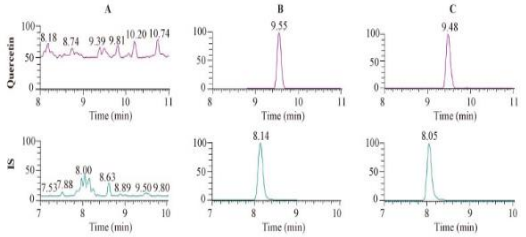

Figure 6: Typical chromatograms of *quercetin* and *naringin (IS)* in rat plasma: (A) blank plasma; (B) blank plasma mixed with *quercetin* and *IS*; (C) plasma samples collected from rats after 1.0 h after being given *quercetin* and *IS*

Table 1: The regression equations, linear range and LLOQs of the five analytes

Compound	Regression equation	r^2	Range (ng/mL)	LLOQ (ng/mL)
Galangin	y=0.0045x+0.003	0.9990	229.8-22984.0	229.8
Galangin-3-methylether	y=0.1317x+0.0017	0.9996	32.0-3200.0	32.0
Kaempferide	y=0.1155x-0.0138	0.9992	78.8-7880.0	78.8
Kaempferol	y=0.0043x-0.0007	0.9996	123.7-12366.0	123.7
Quercetin	y=0.0083x-0.0033	0.9990	137.8-13780.0	137.8

y = peak-area ratios of analytes to IS; x = concentration of analytes in plasma (ng/mL), LLOQ = low limits of quantification

Table 2: *Intra* and *inter* day precision and accuracy for the determination of the five analytes from the assay samples

Compound	Spiked conc.(ng/mL)	Intra-day (n = 3)			Inter-day (n = 5)		
		Mean conc. (ng/mL)	*Precision (RSD, %)*	*Accuracy (RE, %)*	*Mean conc. (ng/mL)*	*Precision (RSD, %)*	*Accuracy (RE, %)*
Galangin	574.6	530.1	2.6	-7.7	526.9	3.3	-8.3
	2298.4	2478.4	3.5	7.8	2521.0	1.2	9.7
	22984.0	23159.9	1.6	0.8	22963.1	1.9	-0.1
Galangin-3-methylether	80.0	86.4	7.4	8.0	85.8	6.1	7.3
	320.0	310.8	4.8	-2.9	312.6	5.4	-2.3
	3200.0	3038.2	1.5	-5.1	3052.4	3.7	-4.6
Kaempferide	197.0	186.3	5.5	-5.4	189.5	3.0	-3.8
	788.0	797.6	4.6	1.2	796.5	2.0	1.1
	7880.0	8020.4	1.6	1.8	8015.3	1.9	1.7
Kaempferol	309.2	314.6	2.0	1.7	316.2	2.7	2.3
	1236.6	1306.0	7.8	5.6	1310.4	4.8	6.0
	12366.0	13453.0	6.8	8.8	13424.8	2.2	8.6
Quercetin	344.5	322.5	4.8	-6.4	326.3	6.2	-5.3
	1378.0	1296.8	2.0	-5.9	1278.4	4.4	-7.2
	13780.0	12074.0	3.1	-12.4	11986.3	1.9	-13.0

Note: conc. = concentration

Recovery

As shown in Table 3, all the extraction recoveries were higher than 88.7 % at three levels. No significant matrix effect was observed and the matrix ratios were in range of 72.9 - 98.8 % for plasma samples.

Pharmacokinetic comparison

The pharmacokinetic process of g*alangin, galangin – 3 - methylether, kaempferide, kaempferol* and *quercetin* in normal and gastric ulcer rats were shown in Figure 3 and Table 4. It was found that the metabolism and transport of the five analytes in gastric ulcer rats were faster than that in normal rats after intra gastric administration of *Shudage - 4* extracts. For *galangin, kaempferide* and *kaempferol,* the pharmacokinetic characteristics of elimination rate constant (K) in normal rats were 9.83 ± 2.08, 29.65 ± 2.30, and 95.87 ± 17.38 (1 / min), whereas these values in gastric ulcer rats were 1.36 ± 0.43, 5.83 ± 1.80, and 40.01 ± 10.52 (1 / min), which were significantly lower (P < 0.01) than the normal group.

The AUC $_{(0–t)}$ values of *galangin – 3 - methylether, kaempferide, kaempferol,* and *Quercetin* in gastric ulcer rats were significantly decline (P < 0.01) to 39.72 ± 4.86, 109.38 ± 8.43, 476.49 ± 26.01, 160.44 ± 2.09 (μg / mL · min) ; The C $_{max}$ values of *kaempferide, kaempferol* and *quercetin* were decline to 0.70 ± 0.08 (P < 0.05), 3.78 ± 0.04 (P < 0.01), 0.88 ± 0.20 (P < 0.01) (μg / mL) respectively.

Table 3: Recovery and matrix effect of the five analytes and IS in rat plasma (mean ± SD, n = 6)

Compound	Spiked conc. (ng/mL)	Recovery (%)	RSD (%)	Matrix effect (%)	RSD (%)
galangin	574.6	88.7±3.9	4.4	90.5±1.9	2.1
	2298.4	102.3±4.1	4.0	72.9±3.0	4.2
	22984.0	99.9±2.7	2.7	87.2±1.4	1.6
galangin-3-methylether	80.0	117.3±1.4	1.2	81.0±1.6	2.0
	320.0	110.1±5.0	4.6	98.8±1.9	2.0
	3200.0	106.5±2.7	2.5	92.8±2.1	2.3
kaempferide	197.0	106.1±2.7	2.6	79.0±0.7	0.9
	788.0	107.4±2.7	2.6	99.7±0.6	0.6
	7880.0	96.9±0.5	0.5	81.2±1.6	2.5
kaempferol	309.2	93.3±3.0	3.2	74.9±1.1	1.5
	1236.6	89.7±0.5	0.7	74.8±3.3	4.4
	12366.0	95.2±3.7	3.8	87.8±1.1	1.3
Quercetin	344.5	107±4.9	4.6	94.4±1.0	1.0
	1378.0	94.9±2.3	2.4	84.9±2.5	2.9
	13780.0	94.7±3.1	3.3	93.7±3.2	3.5

conc. = concentration

Table 4: Pharmacokinetic parameters for the five analytes in normal and model rats after oral administration of *Shudage - 4* extract (mean ± SD, n = 6)

Compound	Group	C_{max}(μg/mL)	$t_{1/2}$ (min)	K (1/min)	$AUC_{(0-t)}$(μg/mL·min)	$AUC_{(0-\infty)}$ (μg/mL·min)
galangin	Normal	0.60±0.02	28.29±2.03	9.83±2.08	73.06±5.85	74.57±6.69
	Model	0.59±0.02	28.65±3.15	1.36±0.43$^{\#\#}$	72.23±5.28	73.85±5.87
galangin-3-methylether	Normal	0.61±0.02	30.56±1.51	37.79±8.62	63.10±2.40	65.01±2.43
	Model	0.46±0.10	36.96±3.25$^{\#}$	12.62±9.14$^{\#}$	39.72±4.86$^{\#\#}$	41.51±5.45$^{\#\#}$
kaempferide	Normal	0.86±0.03	41.71±15.64	29.65±2.30	148.96±3.99	190.63±5.76
	Model	0.70±0.08$^{\#}$	64.55±4.22	5.83±1.80$^{\#\#}$	109.38±8.43$^{\#\#}$	168.86±5.61$^{\#\#}$
kaempferol	Normal	4.29±0.07	44.36±13.35	95.87±17.38	609.02±16.84	637.59±28.83
	Model	3.78±0.04$^{\#\#}$	43.90±2.80	40.01±10.52$^{\#\#}$	476.49±26.01$^{\#\#}$	494.51±24.81$^{\#\#}$
quercetin	Normal	1.07±0.03	41.95±16.08	12.19±2.56	208.23±8.78	308.40±70.71
	Model	0.88±0.20$^{\#\#}$	55.806±3.394	7.61±3.17	160.44±2.09$^{\#\#}$	282.47±62.00

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (compared with normal group)

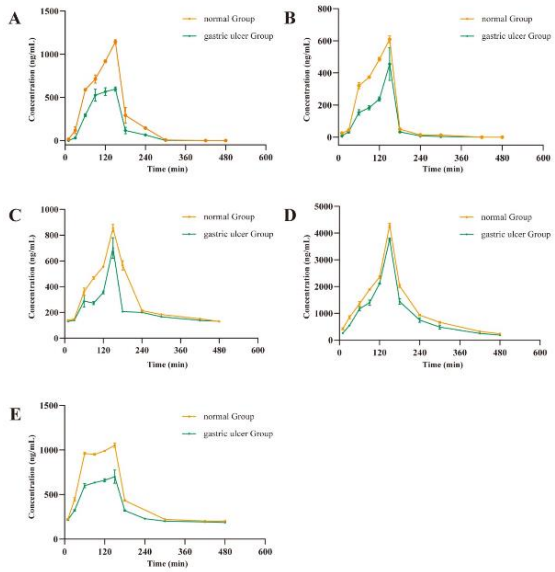

Figure 7: Mean plasma concentration – time curves of the five analytes in normal and gastric ulcer rats after oral administration of *Shudage-4* extract. (A) galangin; (B) galangin–3-methylether; (C) kaempferide; (D) kaempferol; (E) quercetin

DISCUSSION

It has been used to treat stomach for thousands of years in Mongolia. A series of studies on the Mongolian medicine *Shudage - 4* has been conducted in the early stage by our groups, and the results of pharmacological experiments show that *Shudage - 4* has a good effect on gastritis and gastric ulcer [13-15]. Phytochemical studies have revealed flavonoids are one of the main ingredients in *Shudage - 4* [6-8]. The total flavonoids extracts of *Shudage - 4* was purify and enriched by AB - 8 macroporous adsorptive resins in our research, and the content of total flavonoids were over 80% [6 - 8]. The contents of *galangin, galangin-3-methylether, kaempferide, kaempferol* and *Quercetin* were all high determined by using HPLC [6-8]. Meanwhile, the five flavonoids ingredients had good pharmacological activities, which could be used as the representative components of *Shudage-4* in the treatment of gastric diseases [13-15].

The pharmacokinetic parameters, including K for *galangin*, $t_{1/2}$, K and AUC for *galangin – 3 - methylether*, C_{max}, K and AUC for *kaempferide* and *kaempferol*, C_{max} and $AUC_{(0-t)}$ for *quercetin* showed significant difference ($P < 0.05$) in the normal and gastric ulcer rats. The metabolism and transport of the Mongolian Medicine *Shudage - 4* in gastric ulcer rats were faster than that in normal rats and one possible reason for these differences may be flavonoids ingredients of *Shudage - 4* concentrated and consumed in the lesion site faster than the normal group. Double peaks of *galangin, galangin – 3 - methylether,* and *quercetin* are shown in Figure 7 A, B and E, and the results might be due to enterohepatic circulation. Flavonoids enter the blood circulation, firstly, they are carried from the liver to the small intestine, which process needs to pass through the bile duct; then be absorbed into blood by the gastrointestinal system [18]. Double-peak phenomenon of quercetin is controversial. According to one study, no double-peak on quercetin [19], which is inconsistent with our results. Analysis of the cause may be due to the drug interactions. Drug interactions may influence the absorption and elimination of flavonoids.

CONCLUSION

The UPLC–ESI–MS/MS-based assay for the determination of *galangin, kaempferide, galangin – 3 - methylether, kaempferol* and *quercetin* at the same time from effective fraction of the Mongolian Medicine *Shudage-4* in plasma has been successfully developed. All results of the methodology met the criteria of the guidelines of Chinese Food and Drug Administration (CFDA). Thus, the method is specific, sensitive, precise

and stable. It was applied to the pharmacokinetic studies of the five flavonoids successfully from effective fraction of Mongolian Medicine *Shudage - 4* in rat plasma. The *in vivo* pharmacokinetic data obtained in rat plasma also suggest the developed method would be suitable for use in research and clinical conditions.

DECLARATIONS

Acknowledgement

This work was supported by the Natural Science Foundation of Inner Mongolia Autonomous Region (the project no. 2019ZD16).

Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We declare that this work was undertaken by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yu Dong did the study design. Xin Jia and Yinfei Du processed the data and were responsible for data collection. Xin Jia and Jia Xu handled manuscript writing. Xin Jia and Yinfei Du contributed equally to this work and should be considered a co-first author.

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