Supporting Information

Antidementia Effect, Metabolic Profiles and Pharmacokinetics of GJ-4, a Crocin-rich Botanical Candidate from *Gardeniae Fructus*

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1. Method validation

Before this method was put into use, it was performed to evaluate the selectivity, linearity and LLOQ, precision and accuracy, recovery, matrix effect and stability in accordance with the Guidance for Industry: FDA Bioanalytical Method Validation (USFDA, 2001).

1.1 Selectivity

Selectivity was evaluated by comparing the chromatograms of blank rat samples, blank plasma mixed with standard solution, and plasma sample obtained at 4 h after oral administration of GJ-4 at a dose of 500 mg/kg/day.

The representative multiple reaction monitoring (MRM) chromatograms of 2 analytes and IS in rat plasma were shown in Fig. S2. There was no endogenous interference observed.

1.2 Linearity of the calibration curve and lower limit of quantification

The linearity with a $1/X^2$ weighting factor was assessed by assaying standard plasma samples at six concentration levels. For calibration curves, the measured peak area ratios (y) of analytes to IS were plotted versus the corresponding nominal concentration (x) of analytes. The lower limit of quantification was determined at a signal-to-noise of 10 by analyzing the standard plasma sample. The deviation of each back-calculated standard concentration was required to be within 15% of the nominal concentration, except for the LLOQ, for which a deviation of 20% was permitted.

The correlation coefficients (r) were 0.9969 for crocetin, 0.9933 for CME (shown in Table S2). The Table S3 showed that the lower limits of quantification (LLOQ) for crocetin and CME were 0.625 and 0.125 ng/mL. Therefore, this method was considered sensitive for the quantification of 2 analytes in rat plasma.

1.3 Precision and accuracy

The intra-day precision and accuracy were evaluated by analyzing six replicates of QC samples (LLOQ, LQC, MQC and HQC) within one day. For inter-day accuracy and precision, four different levels of QC samples (LLOQ, LQC, MQC and HQC) were evaluated in 6 batches on three separate days. The acceptability criterion for accuracy was a deviation within 15% (relative error, RE), and for precision, it did not exceed

15% (relative standard deviation, RSD).

The values of intra- and inter-day precision and accuracy at three concentrations (LQC, MQC, HQC) were shown in Table S4**.** The intra-day precision (RSD %) of the analytes was less than 8.18%, and the accuracy (RE %) of them ranged from 0.03 to 12.50%. Similarly, the inter-day precision (RSD %) of them were less than 7.48% and the accuracy (RE %) ranged from -3.07 to 6.69%. The precision and accuracy of this method were satisfactory.

1.4 Extraction recovery and matrix effect

The evaluations of the recovery and matrix effect of the two analytes were conducted at LQC, MQC and HQC levels in six replicates, and IS of them was determined at 300 ng/mL. The extraction recoveries of the two analytes and IS were measured by comparing the peak areas of extracted plasma standards with those of extracted blank plasma spiked at the corresponding concentrations. Six blank plasma samples from different rats were used to investigate the matrix effect. The matrix effect was assessed through the comparison of the peak areas of two analytes and IS spiked in extracted blank plasma with those of the standard solution at corresponding concentrations, and the ratio of peak area was regarded as the matrix effect.

The recoveries and matrix effects of the two analytes were presented in Table S5. The recoveries of the two analytes ranged from 85.3 to 96.2% at three concentrations (LQC, MQC, HQC) and the recovery of IS was 91.5%, illustrating consistent recovery and precision. Similarly, the matrix effects ranged from 86.1 to 96.2% and the matrix effect of IS was 95.5%, which demonstrated that no significant influence of matrix effect was detected in rat plasma.

1.5 Stability

The stability of the two analytes in rat plasma was assessed by measuring six replicates of plasma samples at three QC levels (LQC, MQC and HQC) under different conditions: after storage at room temperature for 4 h, in an autosampler for 24 h at 8 ℃ after processing, after three freeze-thaw cycles, after 30 days stocked at -80 ℃ to room temperature. Stability results should be within 15% of the nominal concentrations.

All data from the stability test were summarized in Table S6 and Table S7. The

results indicated that the two analytes in rat plasma were stable at 25 ℃ within 4 h (short term stability), in an autosampler within 24 h at 8 ℃ after processing (auto-stability), after three freeze-thaw cycles (-80 ℃ to room temperature, freeze-thaw stability), and at -80 ℃ within 30 days (long term stability). Hence, the stability test proved that the two analytes were stable under routine laboratory conditions.

Table list:

Table S1 The multiple reaction parameters of the two analytes

Name	t_R (min)	Parent ion	Daughter ion	Cone Voltages(V)	Collision Energy(V)	Dwells
crocetin	3.07	329.17	293.15	30	10	0.005
Crocetin monomethyl ester (CME)	4.57	343.19	293.15	30	10	0.005
IS	4.00	351.25	137.06	30	30	0.005

Table S2 The regression equations, linear ranges, LLOQ of the two analytes (n=3)

Analytes	Linear range(ng/mL)	Regression equation	r	LLOQ (ng/mL)
crocetin	0.625-240	y=0.0460365x+0.00305648	0.9969	0.625

CME	0.125-160	y=0.096069x+0.00624339	0.9933	0.125

Table S3 Precision and accuracy data of the two analytes at LLOQ concentration (n=6).

Analytes	Spiked conc.(ng/mL)	Mean	RSD(%)	RE(%)
crocetin	0.625	0.617	5.98	-1.28
CME	0.125	0.129	7.52	3.20

Table S4 Intra-day and inter-day precision and accuracy of the two analytes at three concentration (LLOQ, LQC. MQC, HQC) (n=6)

Analytes	Spiked conc (ng/ml)	Intra-day (n=6)			Inter-day (n=18)		
		Observed conc.(ng/ml)	Precision (RSD, %)	Accuracy (RE, %)	Observed conc.(ng/ml)	Precision (RSD, %)	Accuracy (RE, %)
crocetin	0.625	0.627	8.18	0.03	0.629	7.22	0.63
	1.25	1.26	6.29	0.80	1.26	5.53	0.89
	45.0	49.2	4.19	9.33	48.0	6.19	6.69
	180	197	4.07	9.40	185	6.77	3.12
CME	0.125	0.119	6.51	4.80	0.121	5.35	-3.07
	0.250	0.264	4.57	5.60	0.258	4.67	3.38
	30.0	33.3	2.19	11.0	31.9	6.15	6.65
	120	135	2.37	12.5	124	7.48	3.37

Table S5 Recoveries and matrix effects of the two analytes and IS (n=6)

Analytes	Spiked conc (ng/ml)	Matrix effect(n=6)		Recovery(n=6)	
		Mean	RSD (%)	Mean	RSD (%)
IS	300	95.5	3.95	91.5	5.95
crocetin	1.25	96.2	8.95	92.8	10.40
	45	89.4	3.01	85.3	3.31
	180	86.5	2.88	96.2	6.14
CME	0.25	89.9	1.95	92.2	7.21
	30	87.9	1.72	91.6	3.01
	120	86.1	0.94	91.5	3.70

Table S6 Short-term stability and auto-stability of the two analytes (n=6)

Analytes	Spiked conc.(ng/mL)	25 ℃ for 4 h			8 ℃ for 24 h		
		Measured conc.(ng/mL)	Precision (RSD, %)	Accuracy (RE, %)	Measured conc.(ng/mL)	Precision (RSD, %)	Accuracy (RE, %)
crocetin	1.25	1.12	0.51	-10.40	1.27	3.89	1.60
	45	43.9	8.58	-2.44	45.7	6.32	1.56
	180	172	6.73	-4.44	170	3.58	-5.56

	0.25	0.238	4.62	-4.80	0.25	2.38	-1.20
CME	30	32.7	4.29	9.00	32.3	1.46	7.67
	120	119	3.01	-0.83	123	4.61	2.50

Table S7 Freeze-thaw stability and long-term stability of the two analytes (n=6)

Analytes	Spiked conc.(ng/mL)	Three-freeze-thaw cycles			Frozen for 1 months		
		Measured conc.(ng/mL)	Precision (RSD, %)	Accuracy (RE, %)	Measured conc.(ng/mL)	Precision (RSD, %)	Accuracy (RE, %)
	1.25	1.26	6.48	0.80	1.24	8.52	0.80
crocetin	45	43.8	5.14	-2.67	44.2	2.79	-1.78
	180	183	9.57	1.67	163	3.48	-9.44
	0.25	0.244	10.93	-2.40	0.235	7.73	-6.00
CME	30	33.1	3.25	10.33	27.3	3.19	-9.00
	120	123	6.50	2.50	110	3.43	-8.33

Figure Captions

Fig. S1 Fragmentation patters of Crocin-3

Fig. S2 Typical MRM chromatograms of (a) Crocetin, (b) Crocetin monomethyl ester, (c) IS in rat plasma. (A) Blank plasma mixed with high concentration analyte and IS, (B) Blank plasma mixed with lower limit of quantitative concentration analytes and IS, (C) Blank plasma.

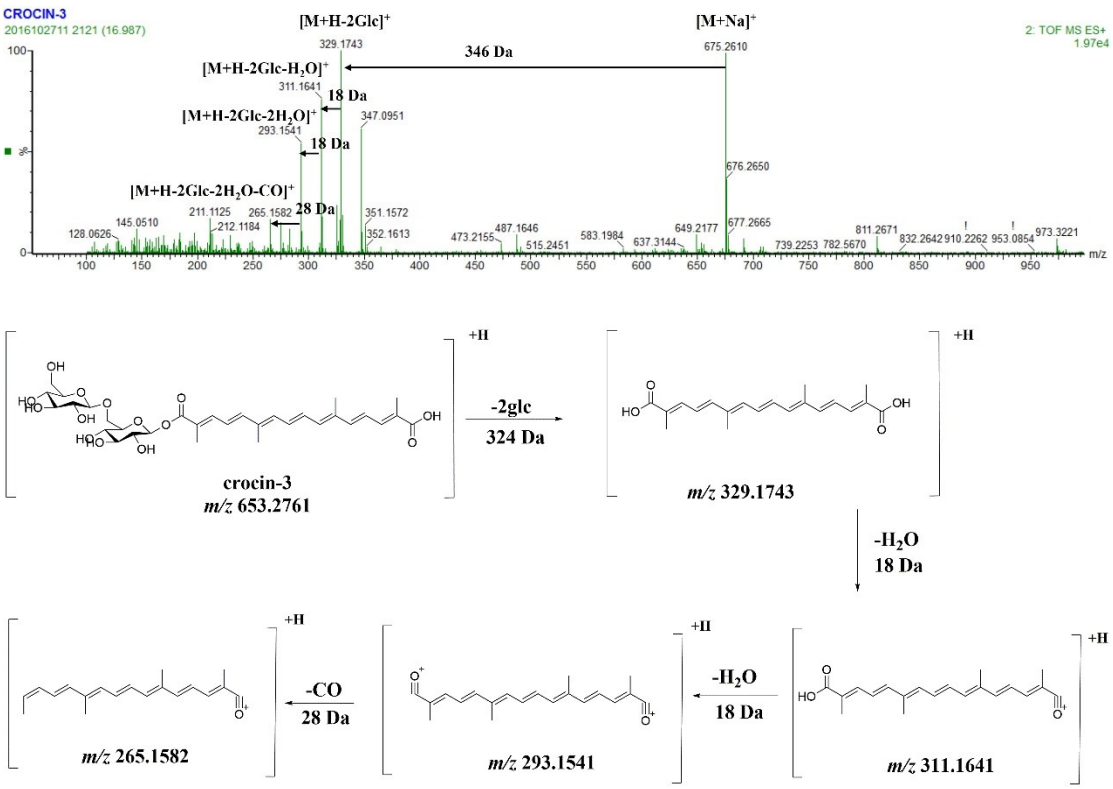

Fig. S1 Fragmentation patterns of crocin-3

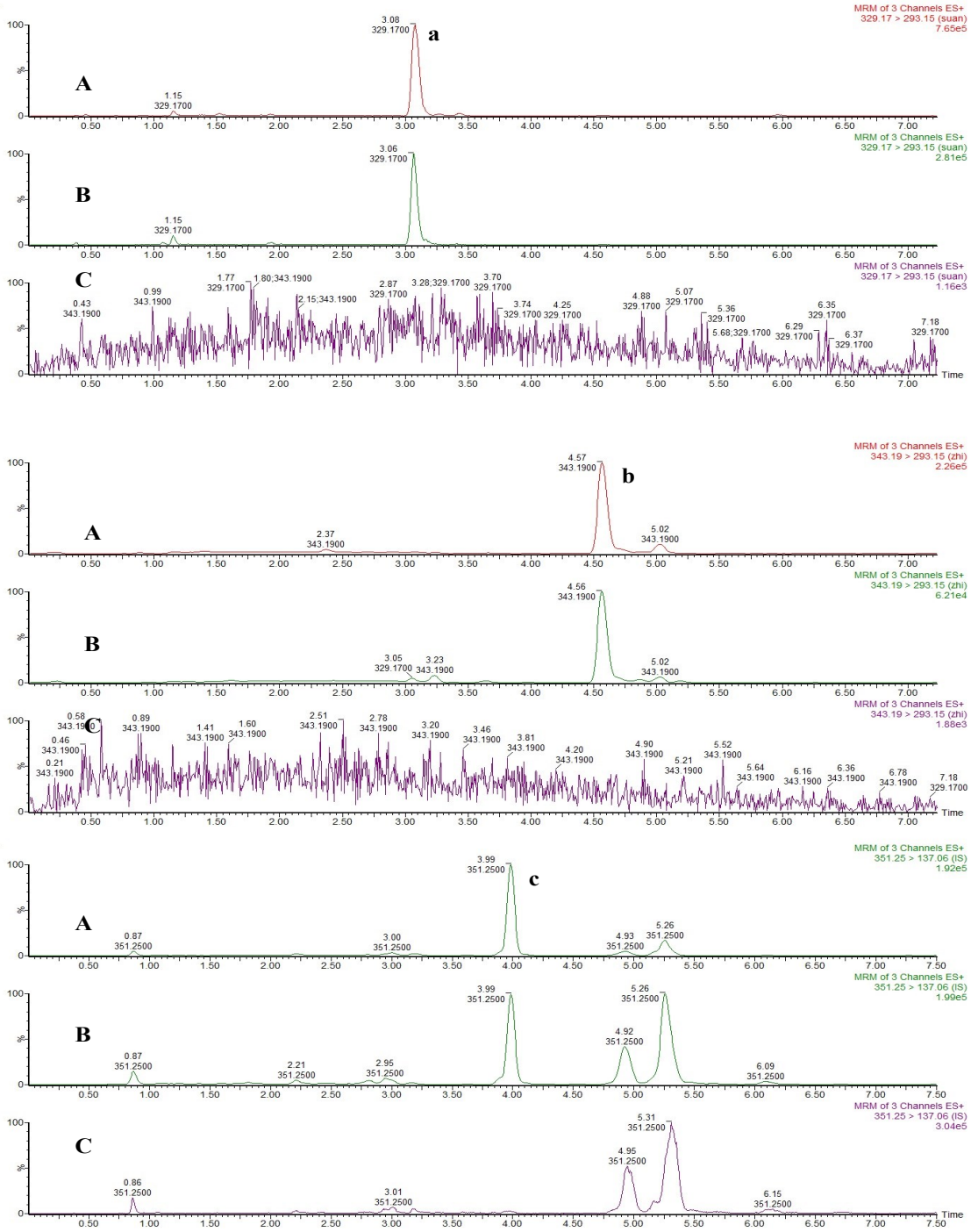

Fig. S2 Typical MRM chromatograms of (a) Crocetin, (b) Crocetin monomethyl ester, (c) IS in rat plasma. (A) Plasma sample obtained at 4 h after oral administration of GJ-4 at a dose of 500 mg/kg/day, (B) Blank plasma mixed with lower limit of quantitative concentration analytes and IS, (C) Blank plasma.