Supplementary Material

Simultaneous determination of paeoniflorin-6′-O-benzene sulfonate (CP-25) and its active paeoniflorin (Pae) metabolite in rat plasma using UPLC–MS/MS: An application for pharmacokinetic and its effects study

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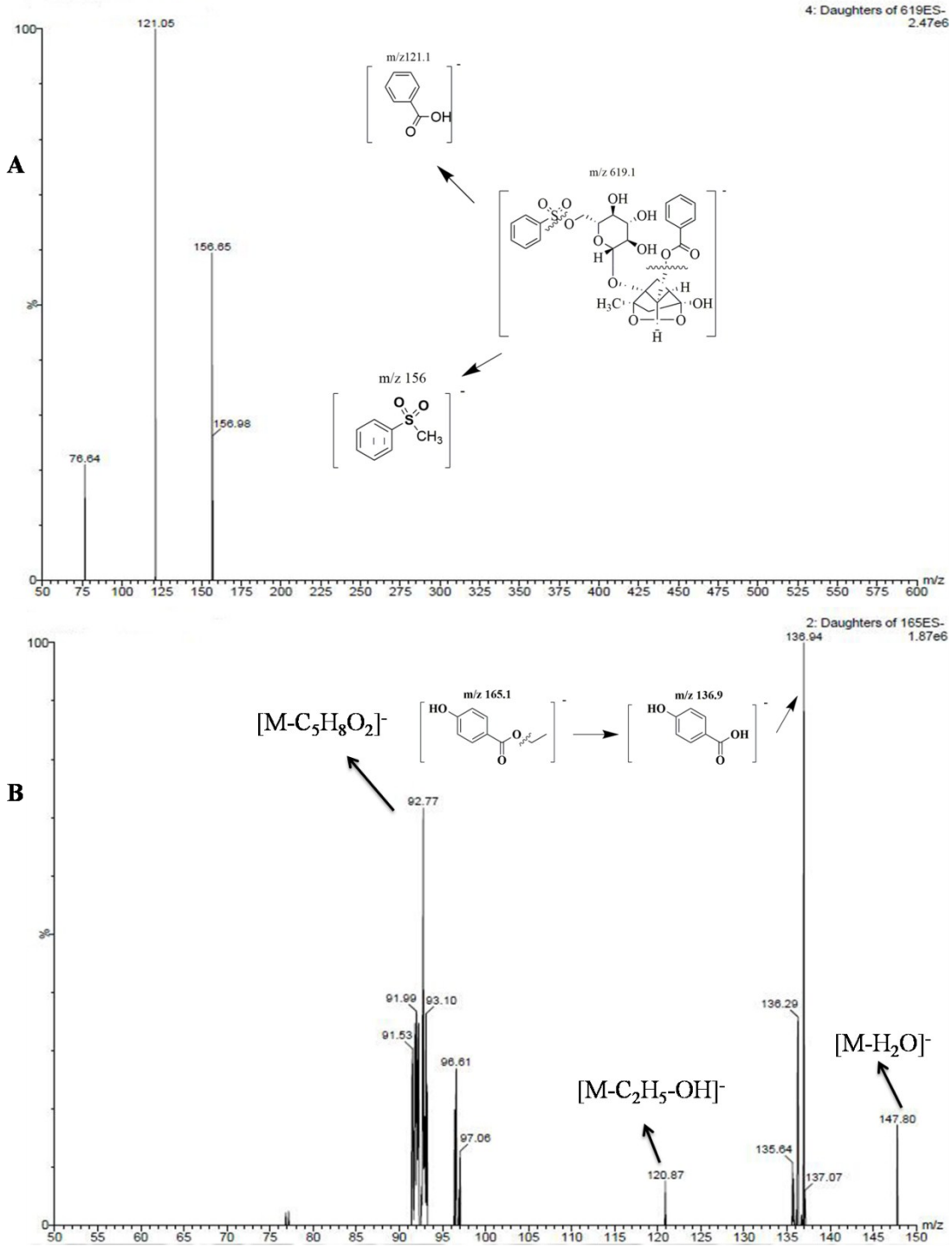
1 Optimization of mass spectrometric and chromatographic conditions

To optimize ESI conditions, CP-25, Pae and IS were dissolved in water/formic acid/methanol (62:0.1:38, v/v/v) and then infused into the mass spectrometer. Though both positive and negative ionization modes were investigated, CP-25, Pae and IS showed higher sensitivity in negative mode than in positive mode. MS parameters included were collision energy, entrance potential and exit potential. Ethyl-P-hydroxybenzoate was chosen as IS because of its similar physicochemical property and chromate graphic behavior with CP-25. Full-scan product ion spectra ion of CP-25, IS and ion of Pae and their fragmentation pathways are shown in Fig. 1. The transitions m/z 619.1→121.1, 479.1→121.1 and 165.1→136.9 in negative electrospray ionization were chosen for quantitation of CP-25, Pae and IS, respectively. Furthermore, the proposed fragmentation chemical structure of CP-25 (A), IS (B) and Pae (C) were shown in Fig. 1. It is noteworthy that CP-25 is an ester derivative of Pae and ester drugs are known to cleave by enzymes in plasma. According to this way, CP-25 needs a period of time to releases Pae (instantaneously or slowly) in plasma. This may be reason that Fig. 1A appear the m/z 156 ion peak and has less fragmentation than Pae (Fig. 1C). Generally, the collision energy has a significant influence on the species and the relative intensity of the production. However, the peak of product ion (m/z 121.1) was strong intensity with same of CP-25 and Pae in different collision energy (50 eV, 16 eV). Considering the high concentration of Pae (M1) in plasma, the responses of CP-25 and Pae under the condition of different collision energy was same strong. Because of the lower collision energy in Pae, we found the m/z 120.3 and m/z 121.3 fragment ion peaks in Fig. 1C, which was adjacent with m/z 121.1 ion peaks. The same phenomenon was also appeared in other papers for Pae research [1-2].

To achieve symmetrical peak shapes and short chromatographic run times, chromatographic elution conditions were optimized. Methanol, not acetonitrile, was selected as part of the mobile phase. The pH condition and the proportion of methanol were investigated. Eventually, the mobile phase system composed of methanol with water containing 0.1% formic acid (pH=3) was selected to achieve well separation

and symmetric peak shapes for CP-25 and IS. ACQUITY UPLC BEH C_{18} column (2.1 mm × 50 mm, 1.7 μm) was selected for the chromatographic separation.



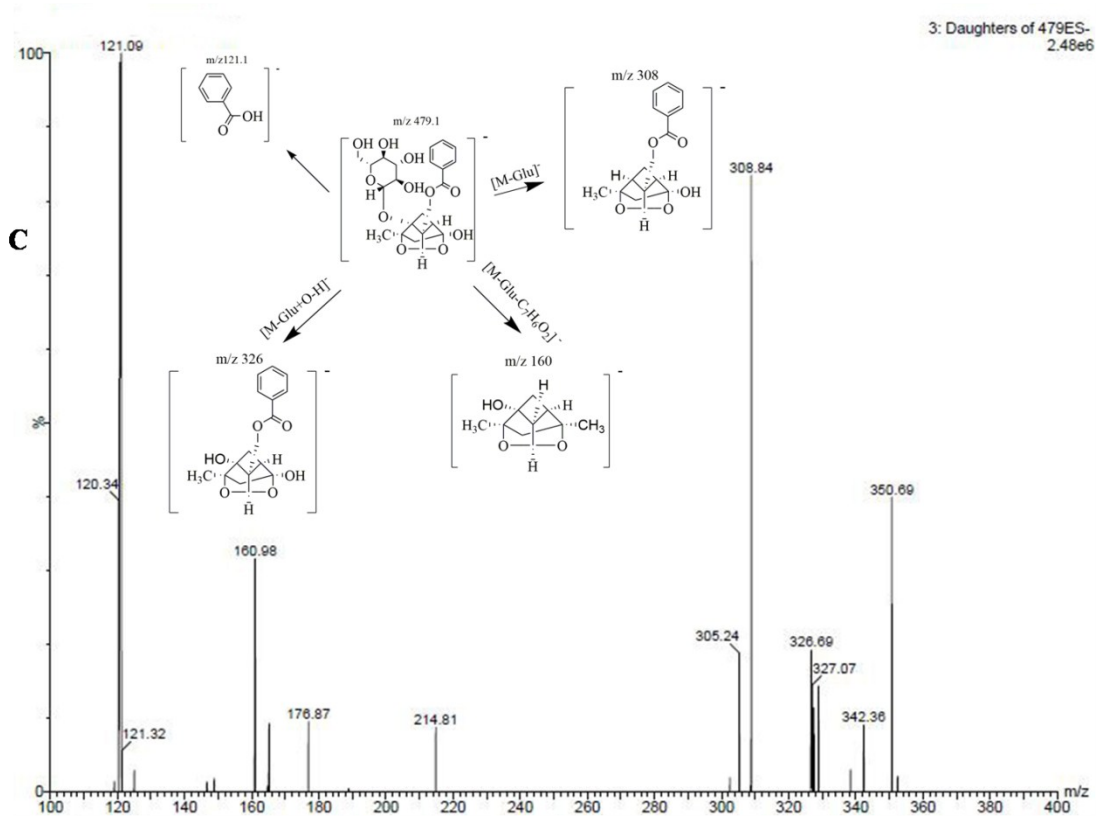


Fig. 1 Full-scan production spectra and the proposed fragmentation chemical structure of CP-25 (A), IS (B) and Pae (C).

2 Method validations

2.1 Specificity

In specificity tests, the blank plasma of rats (blank plasma obtained from six different gender rats) and blank plasma samples spiked with CP-25 and IS, and plasma samples after oral doses of CP-25 were compared. Pae was also evaluated using the same method.

Particular chromatograms of blank plasma (Fig. 2A), blank plasma spiked with CP-25 and IS (Fig. 2B), actual plasma sample after oral administration of CP-25 (Fig. 2C), representative chromatogram of extracted plasma LLOQ sample of CP-25 (Fig. 2D), representative chromatogram of extracted plasma LLOQ sample of Pae (Fig. 2E), blank plasma spiked with Pae and IS (Fig. 2F), and actual plasma sample after oral administration of CP-25 (Pae, M1, Fig. 2G) are represented in Fig. 3. As shown, there is no interference of endogenous peak areas at retention time of CP-25, Pae and IS

(1.84, 0.46 and 1.49 min, respectively).

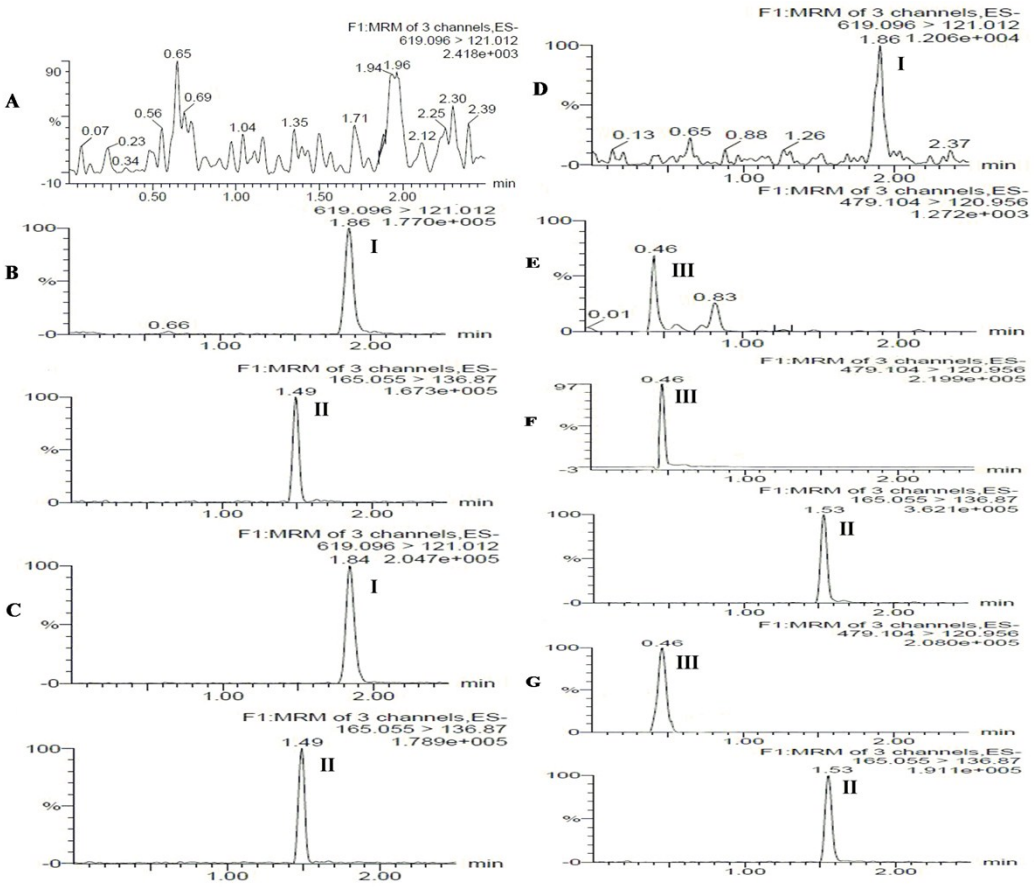


Fig. 2 The particular chromatograms of blank plasma (A); blank plasma spiked with CP-25 and IS (B); 2h after oral administration of CP-25 (50mg·kg^{-1}) in rats (C); bland plasma spiked with CP-25 at LLOQ level (D); bland plasma spiked with Pae at LLOQ level (E); blank plasma spiked with Pae and IS (F) and 2h after oral administration of CP-25 (Pae, M1, 50 mg·kg^{-1}) in rat (G). I: CP-25; II: IS; III: Pae.

2.2 Linearity and sensitivity

For linearity, the stock solution was diluted with methanol to give final concentrations of 2, 5, 10, 50, 100, 200, 400, and 800 ng·mL^{-1} in rats plasma. The IS working solution (200 ng·mL^{-1}) was made from the stock solution using methanol as a diluent. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of QC samples to IS versus the nominal concentration (x) of analytes. The equation of calibration curves was weighted by ln x. The lower limit of quantification

(LLOQ) was determined based on a signal-to-noise ratio of 10:1.

The calibration curves ranged from 2 to 800 ng·mL^{-1} using seven calibration standards with an ln x weighting. The regression equation for calibration curve were y = 0.0056x - 0.0122 (r^2= 0.9990) for CP-25 and y = 0.0049x - 0.0148 (r^2= 0.9990) for Pae respectively. The LLOQ samples (2 ng·mL^{-1}, n=6) were validated with RE within ±10 % and RSD no more than 20 % for each LLOQ sample (Fig. 2D, Fig 2E). The LLOQ of CP-25 in plasma was 2 ng·mL^{-1}, which are sufficient for rat pharmacokinetic studies following oral administration of CP-25.

2.3 Precision and accuracy

The precision and accuracy were calculated by determining QC samples on the same day and three consecutive days, respectively. The relative standard deviation (RSD) was used to express the precision and the accuracy was determined as the percentage of deviation (relative error, RE %) between the measured and nominal concentrations. Intra-day precision, inter-day precision, and accuracy for CP-25 and Pae are summarized in Table 1. All results for the samples tested were within the acceptable criteria of ±15 %, which was included the LLOQ samples (2 ng·mL^{-1}).

Table 1 Summary of inter-, intra-day precision and accuracy data for assays of CP-25 and Pae (n = 5).

Analytes	Spiked (ng·mL^{-1})	Inter-day			Intra-day		
		Mean ± SD	Precision (RSD, %)	Relative error (RE, %)	Mean ± SD	Precision (RSD, %)	Relative error (RE, %)
CP-25	2	2.2±0.3	10.0	5.5	2.1±0.2	10.8	6.1
	5	5.2±0.4	7.3	4.8	5.1±0.4	6.7	2.6
	100	109.3±3.6	3.3	9.31	108.5±4.6	4.2	8.5
	400	411.6±4.5	1.1	2.91	407.6±13.9	3.4	1.9
Pae	2	2.2±0.1	5.3	9.5	2.2±0.1	5.9	11.7

5	5.4±0.3	5.9	8.9	5.2±0.6	12.1	4.5
100	100.9±4.2	4.2	0.9	101.5±9.2	9.1	1.4
400	400.7±39.3	9.8	0.2	404.6±35.6	8.8	1.1

2.4 Recovery and matrix effect

The peak areas of QC samples were obtained in deionized water as A, the corresponding peak areas for analytes spiked after extraction into plasma extracts as B, and peak areas for CP-25 spiked before extraction as C. The recovery and matrix effect values were then calculated as follows:

$$Recovery\ (\%) = \frac{B}{A} \times 100$$

$$Matrix\ effect\ (\%) = \frac{B}{C} \times 100$$

The recovery and matrix effect values of IS (200 ng·mL^{-1}) were also evaluated using the same method [3].

The extraction recovery of CP-25 and Pae in rat plasma at three levels of QC samples ranged from 88.5 % to 102.9 % and 99.5 % to 112.4%. The IS (200 ng·mL^{-1}) was 103.0 ± 3.3 % (Table 2). It is illustrated that the method could meet the requirements of analysis. The matrix effect of CP-25 at three QC concentrations was between 100.9 % and 113.7 % and Pae was between 102.3% and 113.8%. The IS (200 ng·mL^{-1}) was 99.7 ± 4.4 % (Table 2). The results showed that there was minor influence of plasma matrix on the analyzed results.

Table 2 Recovery and matrix effects study of CP-25, Pae and IS in rat plasma (n=5).

Analytes	Spiked (ng·mL^{-1})	Recovery		Matrix effects	
		Mean (%) ±SD	RSD (%)	Mean (%) ±SD	RSD (%)
CP-25	5	102.9±3.3	3.2	113.7±3.2	2.8
	100	89.7±1.6	1.8	109.8±3.9	3.6
	400	88.5±1.4	1.6	100.9±1.8	1.8
Pae	5	112.4±11.0	9.8	109.9±10.2	9.3

	100	101.2±7.4	7.3	113.8±9.5	8.4
	400	99.5±2.7	2.7	102.3±3.1	3.0
IS	200	103.0±3.3	3.2	99.7±4.4	4.4

2.5 Stability

The stability test was evaluated by analyzing QC samples for short-term, post-treatment and three cycles of freeze-thaws of the analyte. The stability was tested by the following conditions: (1) short-term stability in rat plasma at room temperature for 24 h; (2) freeze–thaw stability in rat plasma through three freeze–thaw cycles at -20°C(-20°C to room temperature as one cycle); (3) long-term stability in rat plasma at -20°C for 30 days.

The CP-25 and Pae was stable in the different storage conditions and bias in concentration was within ± 15 % of QC values (Table 3). The results indicated that the method was suitable for the pharmacokinetic study.

Table 3 Stability of CP-25 and Pae in rat plasma (n=5).

Analytes	Spiked ($ng \cdot mL^{-1}$)	Room temperature stability (24h)			Freeze–thaw stability (-20°C, three times)			Long-term stability (-20°C, 30 days)		
		Mean ±SD	RSD (%)	RE (%)	Mean ±SD	RSD (%)	RE (%)	Mean ±SD	RSD (%)	RE (%)
CP-25	5	5.1±0.2	4.5	2.3	5.2±0.5	10.2	4.2	5.5±0.3	6.0	9.7
	100	106.5±8.2	7.7	6.5	100.4±5.0	4.9	4.3	108.7±5.3	4.9	8.7
	400	406.7±5.4	1.3	1.7	393.1±9.1	2.3	-1.7	408.9±19.9	4.9	2.2
Pae	5	5.1±0.3	6.3	2.7	4.8±0.4	8.1	-4.4	5.2±0.2	4.6	4.7
	100	107.0±7.0	6.6	7.0	103.6±6.9	6.7	3.6	99.9±2.5	2.5	-0.1
	400	410.6±22.1	5.39	2.7	397.8±19.7	5.0	-0.6	402.3±30.4	7.5	0.6

Notes and references

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