

jugular vein of pentobarbital-anesthetized rat towards the right atrium and perfused with perfusion fluid. A flexible wire mesh on the probe is sutured to the pectoral muscle. Inlet and outlet lines to the probe are housed within a single piece of flexible tubing, which is externalized by use of a surgical introducer needle. After the surgery, the rats were allowed to recover for two days in single cages under standard conditions (12 h light/dark cycle, a controlled ambient temperature of $22 \pm 2^\circ\text{C}$ and a relative humidity of $60 \pm 10\%$), with free access to food and water.

In vivo microdialysis experiment

Twelve rats were randomly divided into two groups ($n = 6$ per group). One group of animals in the conscious condition was given the aqueous extract of Fructus Corni intra-gastrically at the dose of 1.8 g/kg for 20 min in rat, while the other group was given the aqueous extract of JZP. At the 20 min after the experiment begun, only food was withdrawn. The same dose and regime of administration was carried on. A microdialysis probe (MD-2310) was inserted into the left jugular vein. The inlet of the probe was attached to a BAS syringe driver (MD-2310, USA) connected with a controller (240V/50Hz MD-1000K, BAS, West Lafayette, IN, USA), filled with ACD solution as perfusion fluid. The vascular microdialysis probe was continuously perfused at a 2.0 $\mu\text{l}/\text{min}$ flow rate. Posterior to this equilibrium period, microdialysate was collected using refrigerated fraction collector (MD-1201, BAS, West Lafayette, IN, USA) into 150 μl vials throughout the experiments. All microdialysis experiments were performed in awake freely moving animals kept in an awake animal caging system (Stand-Alone Return and Rodent Bowl kit, BAS, West Lafayette, IN, USA) with no anesthesia used throughout the experiment. Microdialysate samples from the blood vessel were collected for an additional 300 min automatically and stored at -20°C for centralized HPLC analysis.

At the end of each washout period, blank dialysates samples were also collected to ensure that no 5-HMF concentration was detected prior to the following administration.

RESULTS AND DISCUSSION

Safety of Fructus Corni aqueous extract

As shown in Table 1, the contents of five heavy metals (arsenic, cadmium, copper, lead, and mercury) in the aqueous extract of Fructus Corni determined by the AAS analysis fell in the range less than the maximum limit (20 ppm or $\mu\text{g}/\text{g}$) as regulated in China Pharmacopeia (2005) and the World Health Organization (WHO). The amount of the five harmful elements in the aqueous extract of Fructus Corni is in the safe range for herbal test use.

Table 1: Content (ppm or $\mu\text{g}/\text{g}$) of five selected toxic metals in the aqueous extract of Fructus Corni

Heavy metals	Mean \pm RSD (%)	Calibration correlation cofactor (%)
Arsenic	-0.1300 \pm 3.02	99.86
Cadmium	0.0200 \pm 0.84	99.75
Lead	-0.2400 \pm 5.96	99.98
Mercury	0.0030 \pm 2.31	99.89
Copper	4.7400 \pm 0.70	99.99

Optimization of chromatographic conditions

Different mobile phase compositions were tested: (1) aqueous–methanol; (2) aqueous–acetonitrile; (3) aqueous phosphoric acid (0.1%, v/v)–acetonitrile phosphoric acid (0.1%, v/v); (4) aqueous ammonium acetate (0.5%, v/v)–acetonitrile. As a result, the combination of aqueous phosphoric acid (0.1%, v/v)–acetonitrile for mobile phase was the best for separation. Furthermore, other chromatographic variables were also optimized including analytical columns (Hanbon Hedera ODS-2, Hanbon Lichrospher C_{18} and Agilent Zorbax Extend C_{18}), the column temperatures (20°C , 25°C , and 30°C) and the flow rates (0.8 ml/min and 1.0 ml/min). Eventually, the optimal separation was achieved on an Agilent Zorbax Extend C_{18} column (250 mm \times 4.6 mm, 5 μm) at a column temperature of 30°C with a flow rate of 1.0 ml/min.

High-performance liquid chromatographic behavior and specificity

Under the above-mentioned conditions, the retention time for 5-HMF was 9.937 min, the separation for dialysis samples was in good condition and there are no co-eluting disturbing peaks in the vicinity of the two peaks on the chromatogram of the blank dialysates. The results were shown in Figure 1.

Calibration curves, limits of detection and quantification

The calibration curves were performed with ten different concentrations in triplicate. The regression equations were calculated in the form of $y = ax + b$, where y and x were peak area and concentration. LOD and LOQ for each analyte were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively. The linear regression equation (linear ranges) was $y = 160.38x + 0.6461$ (0.376–3.015 mg/L, 5-HMF). The 5-HMF in dialysate showed good linearity ($r^2 = 0.9999$). The LOD and LOQ of the 5-HMF were 0.010 mg/l and 0.376 mg/l, respectively.

Precision and stability

Intra-day and inter-day variations were chosen to determine the precision of the developed method by analyzing certain concentrations of standard solution (at 0.376, 0.753, and

3.015 mg/l). For intra-day variation, the standard solution was analyzed for six times within one day, the inter-day variation was determined in three consecutive days. The results were shown that in Table 2.

Stability study was performed with sample solution at room temperature and analyzed at 0 h, 2 h, 4 h, 8 h, 12 h, 24 h, and 48 h within two days, respectively. Variations were expressed by relative standard deviations (R.S.D.). The R.S.D. of stability was not more than 1.45% for all analytes.

Recovery

An appropriate amount of blank sample was weighed and spiked with known amount of standard solution at concentrations of 0.376, 0.753, and 3.015 mg/l. They were then treated and analyzed as described above. Each concentration was analyzed for six times. The total amount of 5-HMF was calculated from the corresponding calibration curve. The overall recoveries lay between 93.47% and 97.91% for 5-HMF with R.S.D. less than 1.36% indicating that the established method was accurate enough for the determination of the 5-HMF in SD rat microdialysate. The results were shown that in Table 3.

Sample analysis

The established method has been successfully applied to determination the concentration of 5-HMF in crude and JZP aqueous extract from microdialysates in freely moving rats. The results showed that the concentrations of 5-HMF of crude drug aqueous extract in SD rat microdialysate, JZP aqueous extract varied significantly. For instance, for

the crude Fructus Corni aqueous extract, the concentration of 5-HMF in microdialysate was 1.4951 $\mu\text{g/l}$, but higher in rat microdialysate after i.g. administration of the aqueous extract of JZP (5.2662 $\mu\text{g/l}$). The variations might result from processing procedures for Fructus Corni. It was reported that processing or heating could drastically increase the content of 5-HMF.

CONCLUSION

Microdialysis sampling technique causing minimal perturbation to physiological processes has been to date extensively used in and greatly contributory to pharmacodynamics, drug disposition, and metabolism researches in which microdialysis sampling is especially of significance in that traditional sampling methods exhibit evidently more disadvantages. Microdialysis technique with exclusive characteristics of high temporal and spatial resolutions is quite suitable for drug delivery research, specifically in the blood. Additionally, microdialysis also makes it feasible to perform real time and on-line experiment on a free lying animal. The study by Mayor and Illum suggested that the use of anesthetics was proposed to a variable extent to attenuate the ability of the gastrointestinal tract clearance.^[16] As a result, a prolonged residence time of gastrointestinal tract formulation in the gastrointestinal tract was obtained.

In this paper, a freely moving rat model with no anesthesia introduced was utilized throughout the experiment in order to deplete the influence of anesthesia on absorption via gastrointestinal tract with access to physiological condition. The application of microdialysis technique and a free lying animal model contributed to employing a concentration experimental design which not only reduced the amount of experimental animal used and experimental cost, but minimum the influence of anatomical and physical differences between individual animals on experimental data.

This valuable information concerning the concentrations of 5-HMF in freely moving rats after i.g. administration of the aqueous extract of crude drug and JZP which can be of great importance for pharmaceutical industry. Furthermore, from the comparison between the contents in a free lying rats after i.g. administration of the crude and processed Fructus Corni, it can be obviously seen that the contents are enormously changed before and after being processed; this preliminarily explains the reason why crude and processed Fructus Corni aqueous extract have traditionally been used for treating different clinical symptoms and expatiates upon the process principium.

However, further studies involving complete characterization

Table 2: Precision of 5-HMF in freely moving rat dialysate sample. Data are expressed as mean \pm S.D. (n = 5)

Added/ mg/l	Intra-day		Inter-day	
	Determined/ mg/l	RSD/%	Determined/ mg/l	RSD/%
0.376	0.370 \pm 0.004	1.080	0.368 \pm 0.005	1.360
0.753	0.739 \pm 0.010	1.350	0.736 \pm 0.012	1.630
3.015	2.995 \pm 0.013	1.20	2.959 \pm 0.057	1.930

Table 3: Recovery of 5-HMF in freely moving rat dialysate sample. Data are expressed as mean \pm S.D. (n = 5)

Added/mg/l	Determined/ mg/l	Recovery/%	RSD/%
0.376	0.369 \pm 0.060	97.910	1.630
0.753	0.732 \pm 0.008	97.110	1.090
3.015	2.818 \pm 0.072	93.470	2.560

and bioassay analysis of 5-HMF in the aqueous extract of crude drug and JZP are necessary. There is ongoing research in this direction in our laboratory which will be reported in a future manuscript. In a word, the method demonstrates a relatively short analysis time, acceptable sensitivity, precision, accuracy, selectivity, recovery, and stability. And to our knowledge, it is in fact the first report of microdialysis sampling coupled with HPLC-DAD for *in vivo* determination and study of the concentrations of 5-HMF in freely lying rats after i.g. administration of the aqueous extract of crude and processed Fructus Corni and it is benefit to have a further exploration on the mechanism of 5-HMF in treatment.

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