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Original Article

Determination of 5-Hydroxymethyl-2-furaldehyde of Crude and Processed *Fructus Corni* in Freely Moving Rats Using *In Vivo* Microdialysis Sampling and Liquid Chromatography

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Abstract

The purpose of this study was to develop a sensitive and fast microdialysis coupled with high-performance liquid chromatographic (HPLC) method for determination of 5-hydroxymethyl-2-furaldehyde (5-HMF) in free-moving rats after i.g. administration of the aqueous extract of crud *Fructus corni* and its processed products of jiuzheng pin (JZP). The concentration of 5-HMF in free-moving rats after i.g. administration of the water extract of *Fructus corni* and JZP was analyzed by microdialysis coupled with HPLC. Results demonstrated that the concentration of 5-HMF in microdialysate was 1.4951μg/l, but higher in rat microdialysate after i.g. administration of the aqueous extract of JZP (5.2662 μg/l). In conclusion, this method is proved to be rapid accurate and simple. Real-time *in vivo* monitoring the concentration of 5-HMF provides the theoretical basis for further explaining the processing mechanism of *F. corni*.

Keywords: Fructus corni 5-HMF HPLC-DAD; Microdialysi.
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1. Introduction

Traditional Chinese Medicines (TCMs) have been used by practitioners for thousands of years. TCMs have been attracting ever-increasing attention for their therapeutic effects to western medicines with few side effects [1, 2]. Although many TCMs have been proven effective by modern pharmacological studies and clinical trials, their bioactive components and the remedial mechanism are still not well understood. So far, it is widely accepted that TCMs are mostly used in combination, and the composite formulae will produce a synergistic effect or antagonistic action. So, the clarification of bioactive ingredients of TCMs needs an integrative method that can make it possible to perform bioactive assay, chemical isolation and

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identification of captured compounds almost simultaneously [3].

Fructus corni is derived from the dry ripe sarcocarp of *Cornus officinalis Sieb*. et *Zucc*. The crude drug and its jiuzheng pin (JZP) are used clinically for nourishing liver and kidney [4]. It has been increasingly receiving much attention as one of the most popular and cherished herbal medicine in clinic in the world and can be used for medicine hygienic food and cosmetic due to its biological and pharmacological actives such as anti-inflammation anti-virus and anti-oxidant [5, 6]. 5-Hydroxymethyl-2-furaldehyde (5-HMF) is an endogenous product found in plants in free or bound forms. It is found in large amounts in processed *F. corni*, from which it is extracted in hot aqueous infusions. Pharmacological studies on the components showed that 5-HMF had good biological activities such as anti-inflammatory activity and bacteriostatic action [7-9]. Recent studies found that 5-HMF of the *F. corni* has great protective effects against CC14-induced liver injury and vascular endothelial cell injur, therefore, it is believed that 5-HMF is an important active composition of the *F. corni* [10]. The researcher also found that 5-HMF in the JZP is much more than 5-HMF in the crude drugs, this proves that steaming can increase the 5-HMF. Besides, the researchers found 5-HMF from the *F. corni* which is boiled in aqueous is three times higher than that from the methanol ultrasonic extraction, but there is little difference in the preparation. It is concluded that heating has great effect on the amount of 5-HMF, however, the researchers has not made the study about how 5-HMF in the aqueous extract of crude *F. corni* and JZP changes in body.

Intra-gastric (i.g.) administration is often considered as a safe and acceptable route of drug delivery, especially for the substances with biological effects on the liver and kidney [11, 12]. Considerable studies have been undertaken on liver and kidney delivery systems. Therefore, a design using a freely moving animal model in the study is also suggested to be employed due to an effect of the anaesthetic agents to a variable degree on the intra-gastric absorption, in order to make the experimental data on blood delivery more conformable to physiological condition.

Recently, microdialysis coupled with HPLC has been developed as one of the most powerful analytical techniques. Microdialysis is a continuous sampling technique (Figure 1), therefore, each sample represents the average concentration of analyte in the blood during the sampling interval. This is compared to taking discrete blood samples, which represent

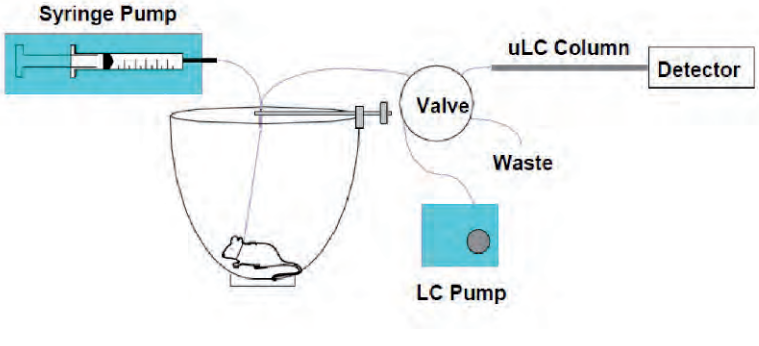


Figure 1. Schematic diagram of microdialysis in freely moving rats.

Table 1. Content (ppm or μg/g) of five selected toxic metals in the aqueous extract of *Fructus corni*.

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

the concentration in the blood only at the time of sampling [13, 14]. Because of the continuous sampling microdialysis is an integrating technique which is less prone to fluctuations than an instantaneous sampling technique. In the present study, microdialysis coupled with HPLC-DAD was utilized to study the concentrations of 5-HMF in freely moving rats after i.g. administration of the aqueous extract of crude drug and JZP. To our knowledge, there have been no reports on the concentration of 5-HMF in freely moving rats using microdialysis coupled with HPLC. A novel method was developed to study the process mechanism of TCM.

2. Materials and methods

2.1. Sample, chemicals and reagents

The processed *F. corni* of jiu zheng pin (JZP) was collected from Henan suppliers. 5-HMF was prepared from our laboratory, and its identity was verified by LC-MS, ^{1}HNMR and ^{13}CNMR [15]. The purity for each standard compound was greater than 98% by HPLC analysis. Acetonitrile was purchased from E. Merck (Darmstadt, Germany). De-ionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Solvents (Millipore Corp., Bedford, MA) were of HPLC grade used for all preparations. All reagents with high grade were obtained from the local market.

2.2. Animals

Twelve male adult Sprague-Dawley rats weighing approximately 300 g were obtained from the Laboratory Animal Center of Zhejiang Chinese Medical University (Zhejiang, China). Animals were acclimatized for at least 5 days with alternating dark/light cycle of each 12 h in a climate controlled room with temperature maintained at 22±1 °C and a relative humidity of 60±10%. Water and standard laboratory food were available ad libitum. All experiments were performed according to the guidelines for the care and use of animals as established by Zhejiang Chinese Medical University.

2.3. Preparation of sample solutions

The powder of *F. corni* and JZP samples quantitatively (20 g) transferred into round-bottomed flask and extracted with 10 times of distilled water for about 2 h and cooled at room temperature. The solid residue was removed by filtration. The residue was boiled again with 10 times of distilled water for 1 h, and after cooling, it was filtered. The final volume was 18 ml.

Atomic absorption spectroscopic (AAS) analysis of five toxic heavy metals, including arsenic, cadmium, chromium, lead and mercury, was preformed on Thermo SOlAARS2 atomic absorption spectrometer with auto-sampler.

2.4. Preparation of ACD solution

Anticoagulant citrate dextrose solution (ACD) consisting of 0.67 g $Na_3C_6H_5O_7.2H_2O$ 0.24 g $C_6H_8O_7.H_2O$, 1.50 g $C_6H_{12}O_6$, was adjusted to pH 7.4, and used as the perfusion medium for microdialysis probes.

2.5. Preparation of standard solutions

A standard stock solution of 5-HMF was prepared by dissolving 6.03 mg of 5-HMF in 10ml of ACD solution for use in sample analysis. The standard stock solution was further diluted with ACD solution to make 7 different concentrations including 1, 1/4, 1/5,

1/4, 3/20, 1/10, 1/20 and 1/40 of the original concentration.

2.6. HPLC analysis

Concentrations of 5-HMF was determined by using Agilent 1200 HPLC system with diode array detector and a chemsatation data analysis system. An Agilent Zorbax Extend C18 column (250×4.6 mm i.d., 5 μm) was utilized as the analytical column and column temperature was maintained at 30 °C. The mobile phase consisted of 2% acetonitrile and 98% of aqueous phosphoric acid (0.1% v/v). The detector wave length was set at 284 nm. All samples were centrifugated at 15000×g for 10 min. An injection volume of 20 μl was used.

2.7. Surgical procedures

Sprague-Dawley rats were anesthetized

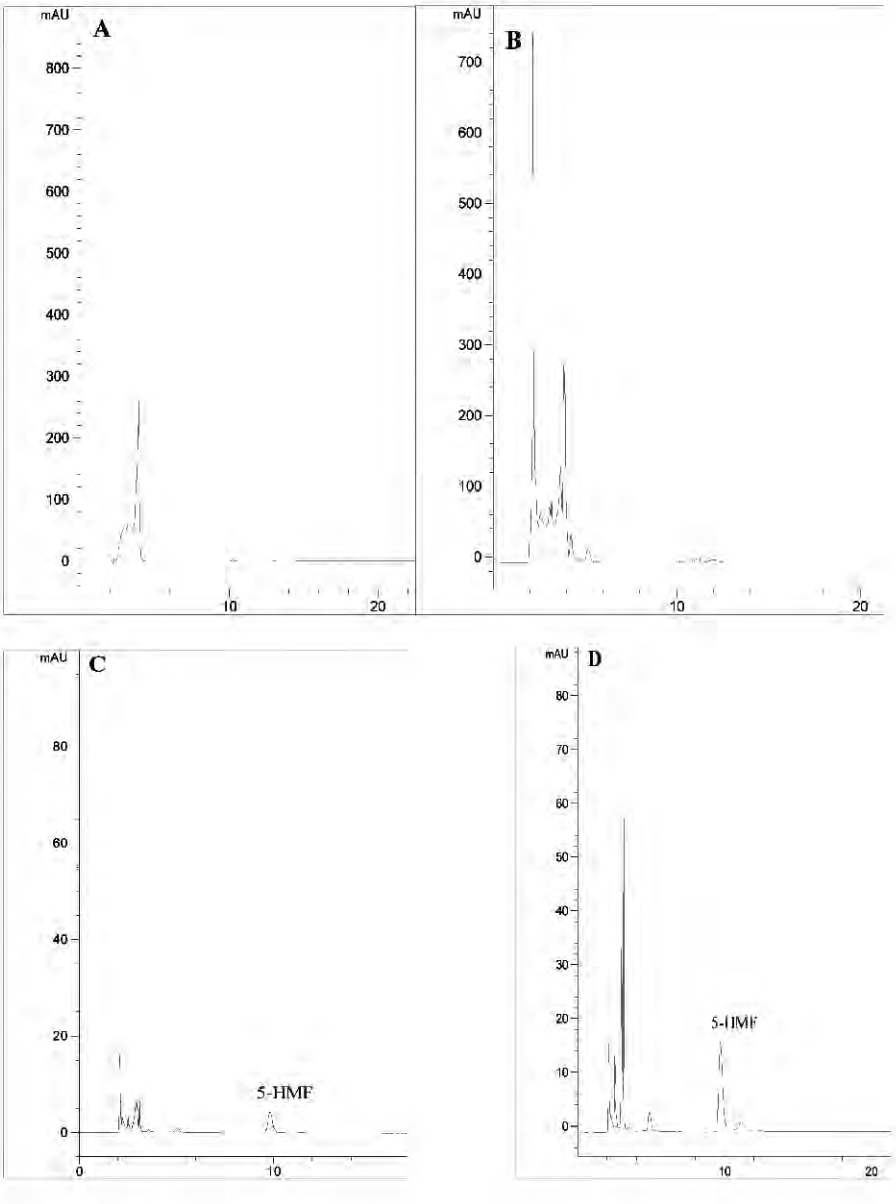
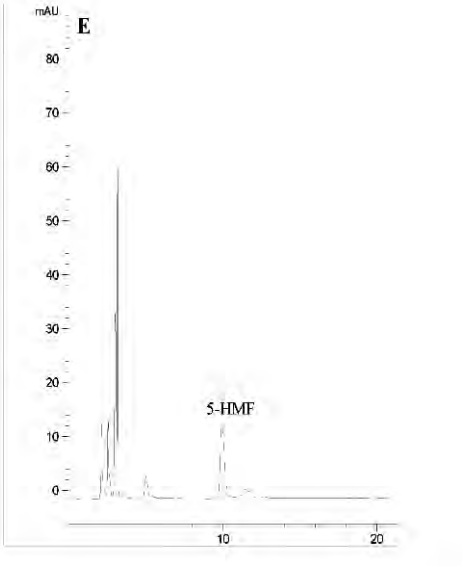


Figure 2. HPLC Chromatograms of 5-HMF in freely moving rat samples.; A. blank ACD solutionusp; B. blank dialysate sample; C. dialysate sample after i.g. administration of the aqueous extract of crud *Fructus corni*; D. dialysate sample after i.g. administration of the aqueous extract of JZP; E. blank dialysate sample with standard 5-HMF.

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

Added/mg.l^{-1}	Intra-day		Inter-day	
	Determined/mg.l^{-1}	RSD/%	Determined/mg.l^{-1}	RSD/%
0.376	0.370±0.004	1.080	0.368±0.005	1.360
0.753	0.739±0.010	1.350	0.736±0.012	1.630
3.015	2.995±0.013	1.20	2.959±0.057	1.930

with ketamine/xylazine (90±10 mg/kg) by intraperitoneal injection and mounted on a stereotaxic frame (Bioanalytical Systems, West Lafayette, IN, USA). A flexible vascular microdialysis probe (MD-2310, BAS, West Lafayette, IN, USA) with characteristics of 0.5 mm O.D., 10 mm membrane length and nominal 18 kDa MW cut-off was inserted into the right jugular vein of pentobarbital-anaesthetized 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 2 days in single cages under standard conditions (12 h light/dark cycle, a controlled ambient temperature of 22±2 °C and a relative humidity of 60±10%), with free access to food and water.



2.8. In-vivo microdialysis experiment

Twelve 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* intragastricly at the dose of 1.8 g/kg while the other group was given the aqueous extract of JZP. At 20 min after the start of the experiment, 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 the perfusion fluid. The vascular microdialysis probe was continuously perfused at a 2.0 µl/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 Raturn and Rodent Bowl kit, BAS, West Lafayette, IN, USA), and no anaesthesia 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.

3. Results

3.1. Safety of F. corni aqueous extract

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

Added/mg.l^{-1}	Determined/mg.l^{-1}	Recovery/%	RSD/%
0.376	0.369±0.060	97.910	1.630
0.753	0.732±0.008	97.110	1.090
3.015	2.818±0.072	93.470	2.560

As shown in Table1, the contents of five heavy metals (arsenic, cadmium, copper, lead, and mercury) in the aqueous extract of *F. corni* determined by the AAS analysis fell in the range less than the maximum limit (20 ppm or μg/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 *F. corni* is in the safe range for herbal test use.

3.2. Optimization of chromatographic conditions

Different mobile phase compositions were tested: (1) aqueous-methanol; (2) aqueous–acetonitrile; (3) aqueous phos-phoric 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 C18 and Agilent Zorbax Extend C18), 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 C18 column (250×4.6 mm 5 m) at a column temperature of 30°C with a flow rate of 1.0 ml/min.

3.3. HPLC behaviour 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 2.

3.4. 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, wh-ere 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 dialys-ate showed good linearity (r^2=0.9999). The LOD and LOQ of the 5-HMF we-re 0.010 mg/l and 0.376 mg/l, respectively.

3.5. 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 1 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, 2, 4, 8, 12, 24 and 48 h within 2 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.

3.6. 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 are shown that in Table 3.

3.7. Sample analysis

The established method has been successfully applied to determine 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 *F. corni* aqueous extract, the concentration of 5-HMF in microdialysate was 1.4951 μg/l, but higher in rat microdialysate after i.g. administration of the aqueous extract of JZP (5.2662 μg/l. The variations might result from processing procedures for *F. corni*. It was reported that processing or heating could drastically increase the content of 5-HMF.

4. Discussion

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 freelying animal. The study by Mayor and Illum suggested that the use of anaesthetics 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 research, a freely moving rat model with no anaesthesia introduced was utilized throughout the experiment in order to deplete the influence of anaesthesia on absorption via gastrointestinal tract, with access to physiological condition. The application of microdialysis technique and a free 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 rats after i.g. administration of the crude and processed *F. 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 *F. corni* aqueous extract have traditionally been used for treating different clinical symptoms and expatiates upon the process principium.

However, further studies involving complete characterization 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, accurately, 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 a free rats after i.g. administration of the aqueous extract of crude and processed *F. corni* and it is beneficial to have further exploration on the mechanism of 5-HMF in treatment.

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References

[1] Wen XD Qi LW Chen J Song Y Yi L Yang XW Li P. Analysis of interaction property of bioactive components in Danggui Buxue decoction with protein by microdialysis coupled with HPLC-DAD-MS. *J Chromatogr B* 2007; 852: 598-604.

[2] Qian ZM Qin SJ Yi L Li P, Wen XD. Binding study of Flos Lonicerae Japonicae with bovine serum albumi-n using centrifugal ultrafil-tration and liquid chromatography. *Biom-ed Chromatogr* 2008; 22: 202-6.

[3] Deterding LJ, Dix K, Burka LT, Tomer KB. On-line coupling of *in vivo* microdialysis with tandem masss pec-trometry. *Anal Chem*1992; 64: 2636-41.

[4] Bellander BM Cantais E Enblad P. Consensus meeting on MD in neurointensive care. *Intensive Care Med* 2004; 30: 2166-9.

[5] Ding X, Wang MY, Yu ZL, Hu W, Cai BC. Studies on separation, appraisal and the biological activity of 5HMF in *Cornus officinalis*. *China J Chin Materia Medica* 2008; 33: 392-484.

[6] Du WF, Cai H, Wang MY, Ding X, Yang H, Cai BC. Simultaneous determination of six active components in crude and processed *Fructus corni* by high performance liquid chromatograp-hy. *J Pharm Biomed Anal* 2008; 48: 194-7.

[7] Xu Q, Li YH, Lü XY. Investigation on influencing factors of 5-HMF content in Schisandra. *J Zhejiang Univ Sci B* 2007; 8: 439-45.

[8] Li YH, Lu XY. Investigation on the origin of 5-HMF in Shengmaiyin decoction by RP-HPLC method. *J Zhejiang Univ Sci* B 2005; 6: 1015-21.

[9] Brustugun J, Tønnesen HH, Edge R, Navaratnam S. Formation and reactivity of free radicals in 5-hydroxymethyl-2-furaldehyde: the effect on isoprenaline photostability. *J Photoch-em Photobiol B* 2005; 79: 109-19.

[10] Graff CL Pollack GM. Nasal drug administration: potential for targeted central nervous system delivery. *J Pharm Sci* 2005; 94: 1187-95.

[11] Hatano T, Yasuhara T, Abe R, Kudat TO. A galloylated monoterpene glucoside and a dimeric hydrolyzable tannin from *Cornus officinalis*. *Phytochemistry* 1990; 29: 2975-8.

[12] Illum L. Nasal drug delivery-possibilities, problems and solutions. *J Controlled Release* 2003; 87: 187-98.

[13] Mayor SH, Illum L. Investigation of the effect of anaesthesia on nasal absorption of insulin in rats. *Int J Pharm* 1997; 149: 123-9.

[14] De Lange ECM, De Boer AG, Breimer DD. Methodological issues in microdialysis sampling for pharmacokinetic studies. *Adv Drug Del Rev* 2000; 45: 125-48.

[15] Tsai TH. Assaying protein unbound drugs using microdialysis techniques. *J Chromatogr B* 2003; 797: 161-73.

[16] Davies MI. A review of microdialysis sampling for pharmacokinetic applications. *Anal Chim Acta* 1999; 379: 227-49.