Short communication

Pharmacokinetics and liver distribution study of unbound curdione and curcumol in rats by microdialysis coupled with rapid resolution liquid chromatography (RRLC) and tandem mass spectrometry

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\textbf{A B S T R A C T}

A sensitive, specific, convenient and endogenous interference-free microdialysis sampling method coupled with RRLC–MS was successfully developed and applied to the determination of protein-unbound curdione and curcumol in biological samples. Microdialysis probes were simultaneously inserted into the jugular vein toward heart and the median lobe near the center of liver of rats under anesthesia. The separation was accomplished on a Zorbax SB-C18 column (2.1 mm × 50 mm, 1.8 μm) with a gradient elution and chromatography was conducted with RRLC system. Analytes were detected by positive ion electrospray tandem mass spectrometry and quantified on the basis of extracted ion chromatography (EIC) peak area signal. The calibration curves were linear over the range of 3.3–213.2 ng/ml for curdione and 8.1–519.2 ng/ml for curcumol. All the validation data, such as accuracy, precision, stability and matrix effect were satisfactory and within the required limits. The validated method was successfully applied to the pharmacokinetic study of curdione and curcumol in rat blood and liver after oral administration of Rhizoma Curcumae extracts. The results could provide a meaningful basis for better understanding of the intracorporal process of Rhizoma Curcumae, which would be helpful for further study both in clinic and laboratory.

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1. Introduction

\textit{Rhizoma Curcumae}, officially listed as blood-activating and stasis-dissolving drug in Chinese Pharmacopoeia [1], has been widely prescribed in clinical practice for the treatment of cardiovascular disease, inflammation, and cancer. Curdione and curcumol are two major bioactive constituents of Rhizoma Curcumae, which demonstrate various pharmacological functions and are commonly used as quality control markers. Curdione has been reported to be the most highly concentrated component of \textit{Rhizoma Curcumae} [2]. It has a potently protective effect on acute liver injury in mice [3] and may be a promising candidate for anti-inflammatory and cancer chemopreventive agents [4]. Curcumol has attracted the attention of researchers due to its excellent anti-cancer effect both in vitro [5] and in vivo [6]. It also may be a potential lead compound for a novel anti-inflammatory drug because of its inhibitory activity on various inflammatory mediators [7]. Thus, for further new drug investigation, it is essential to study the pharmacokinetic profiles of these two bioactive substances.

Currently, several methods had been reported for the determination of curdione and curcumol individually in plasma samples, including GC–FID [8], LC–MS [9,10] and LC–MS/MS [11]. Two studies addressing pharmacokinetic [12] and tissue distribution [13] of the essential oil of \textit{Rhizoma Curcumae} reported the simultaneous analysis of curdione and curcumol in rat by GC. The comparison of these approaches was summarized in Table 1. Unfortunately, all these reports described the total concentration of curdione and curcumol in biological fluid. However, for drugs in vivo, only the free fraction is the therapeutically active portion. What’s more, the conventional sample preparation approaches are laborious and time-consuming. Considering that both curdione and curcumol are volatiles,
instability may easily occur during these complex processes. And as efficiency and speed of analysis has become of great importance in the field of bio-analysis, it is very important to increase the throughput and reduce the analysis costs. RRLC–MS coupled with multiple sites microdialysis gives new possibilities in this area.

Therefore, we now report the development and validation of a novel method based on multiple sites microdialysis combined with RRLC–MS for the simultaneous determination of curdione and curcumol. This sensitive and convenient method has been successfully applied to the in vivo pharmacokinetic study of unbound curdione and curcumol in rat blood and liver, which could help to gain more accurate pharmacokinetic parameters and better clarify the intracorporeal process of Rhizoma Curcumae. To the best of our knowledge, the established method has not yet been reported.

2. Experimental

2.1. Chemicals and reagents

The details of the reference standards, their purity and other materials used in this study were given in Supplementary data.

2.2. Preparation of Rhizoma Curcumae extract

Rhizoma Curcumae was collected from Yueqing, Zhejiang Province, China. The crude pieces of Rhizoma Curcumae were prepared according to the Chinese Pharmacopeia [1]. Fifty grams of crude pieces were immersed into 90% ethanol (1:15, w/v) for 1 h and then extracted twice by electric jacket with a reflux condenser for 1 h each time. The extracts were combined and evaporated by rotary evaporation water vacuum at 40 °C to let the concentration to be 1.4 g/mL of crude drug. The extract was analyzed by HPLC-DAD method established and validated by our laboratory [14]. The contents of curdione, curcumol, germacrone, furanodiene, β-elemene, bisdemethoxycurcumin, demethoxycurcumin and curcumin were 0.211%, 0.079%, 0.065%, 0.017%, 0.019%, 0.0005%, 0.0014% and 0.0079%, respectively.

2.3. High-performance liquid chromatography and tandem mass spectrometry conditions

Chromatographic analysis was performed on an Agilent 1200 system (Agilent, USA) equipped with a binary pump and a thermostatically controlled apartment. Separation was achieved by an Agilent Zorbax-C18 microbore column (2.1 mm × 50 mm, 1.8 μm) at 30 °C. Mobile phase A and B were acetonitrile and water, respectively, both containing 0.05% formic acid. Gradient elution was set as follows: 0–5 min, 40–45% A; 5–8 min, 45–50% A; 8–9 min, 50–55% A; 9–10 min, 55–100% A; 10–15 min, 100% A. The flow rate was 0.4 mL/min. The injection volume was 20 μL. Mass spectrometric detection was carried out by a single quadrupole mass spectrometer (Product No. G2710BA, Agilent Corp, Palo Alto, CA, USA) equipped with an ESI source. The optimized mass of the mass spectrometry were as follows: drying gas (N2), 8 L/min; drying gas temperature, 300 °C; capillary temperature, 350 °C; nebulizing gas pressure(N2), 35 psi; capillary voltage, 3000 V; the optimized decluster potential (DP) for curdione and curcumol were 100 and 70 V, respectively. Analysis was carried out in positive ion mode at the m/z 237.3 for curdione and m/z 219.2 for curcumol by Chemstation software (Agilent Technologies, USA).

2.4. Preparation of stock solutions, calibration standards, and quality control (QC) samples

Stock standard solutions of curdione (0.53 mg/mL) and curcumol (0.65 mg/mL) were separately prepared in methanol, respectively. Calibration standards were prepared by serial dilution of stock solutions with ACD solution to get the concentrations of 3.33, 13.33, 26.65, 53.30, 106.60, 213.20 ng/mL for curdione and 8.11, 32.45, 64.90, 129.80, 259.60, 519.20 ng/mL for curcumol. QC samples were prepared in a similar manner at concentrations of 6.66, 26.65 and 191.88 ng/mL for curdione and 16.23, 64.90 and 467.28 ng/mL for curcumol.

2.5. Method validation

The analytical method was validated according to the criteria suggested by the USFDA [15]. The calibration curves were constructed using six standards ranged from 3.33 to 213.20 ng/mL for curdione and 8.11 to 519.20 ng/mL for curcumol. The intra–day precision and accuracy of the method were determined by assaying five replicates of QC samples in a single day while its inter–day precision and accuracy were estimated by analyzing triplicate of QC samples in five consecutive days. The stability was evaluated by analyzing QC samples in three conditions, i.e. freeze–thaw for three cycles, room temperature for 24 h and storage in freezer at −20 °C for 2 days.

2.6. Animal, microdialysis experiment and drug administration

Adult, male Sprague-Dawley rats (n=11, 250–300 g) were obtained from the Laboratory Animal Center of Nanjing University of Chinese Medicine. These animals were specifically pathogen-free and allowed to acclimate to their environmentally controlled quarters (24 ± 1 °C, 55% humidity and 12:12 light–dark cycle). Animal welfare and experimental procedures strictly conformed to the Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996) and the related ethics regulations of Nanjing University of Chinese Medicine.
Before surgery, the rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) and remained anesthetized throughout the experimental period. The blood (10 mm in length) and liver (4 mm in length) microdialysis probes with a molecular weight cut-off of 15 kDa (Microbiotech/se AB, Stockholm, Sweden) were positioned within the jugular vein toward the right atrium and the median lobe near the center of the liver, respectively. The microdialysis systems were perfused with ACD solution at a flow-rate of 2.0 μL/min by a syringe pump (CMA/400, Stockholm, Sweden). The rat temperature was maintained at 37 °C using a heating pad during the experiment.

After a 2.0 h post-surgical stabilization period subsequent to the successful implantation of probes, the extract of *Rhizoma Curcumae* was administered orally (14.17 g/kg, consists of curdione and curcumol 29.77 mg/kg and 11.20 mg/kg, respectively). The microdialysis samples were collected every 30 min for each probe by a refrigerated fraction collector (CMA/470). The collected samples were kept at −20 °C and analyzed by LC–MS within 48 h.

### 2.7 Recovery of microdialysis probes

The in vitro recoveries of probes were determined prior to the in vivo calibration by gain and by loss method [16], respectively. For in vivo recovery determinations, a retrodialysis calibration technique was utilized [16]. Details of validation procedure are given in Supplementary data.

### 2.8 Statistical analysis and pharmacokinetic application

Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic program WinNonlin Standard Edition Version 5.2 (Pharsight, Mountain View, CA, USA) by non-compartmental method.

The results are presented as mean ± standard deviation of each treatment. Statistics were analyzed by SPSS16.0 program (SPSS Inc., Chicago, IL, USA).

### 3. Results and discussion

#### 3.1 Method validation

Typical chromatograms of blank dialysates, standard solutions and microdialysis samples were shown in Fig. 1. The retention times of curdione and curcumol were 3.9 and 5.9 min, respectively. No interfering peak from endogenous compounds was observed in blank dialysates. The average values of matrix effects were 91.5% (RSD = 4.9%, n = 5), 103.4% (RSD = 1.8%, n = 5) and 108.4% (RSD = 2.0%, n = 5) for curdione and 92.8% (RSD = 6.6%, n = 5), 99.0% (RSD = 6.9%, n = 5) and 108.1% (RSD = 2.0%, n = 5) for curcumol, indicating that the matrix effects from salts and endogenous compounds could be negligible for the present method. The regression equations were \( Y = 13.307X + 38.902 \) \( (r = 0.9989) \) for curdione and \( Y = 962.56X - 3832.6 \) \( (r = 0.9986) \) for curcumol \( (Y \) represents the peak area and \( X \) represents the concentration). The LLOQ for curdione and curcumol were 3.33 and 8.11 ng/mL, respectively. Both precision and accuracy were acceptable for bio-analysis (Supplementary Table S1). The stability results indicated that both curdione and curcumol were stable during microdialyses storage and analytical process (Supplementary Table S2).

The results of in vitro recovery calibration suggested that there was no significant difference between the gain and loss method (Supplementary Figure S1). Therefore the retrodialysis method can be used for in vivo recovery calibration. The in vivo recoveries of blood probes were 68.19 ± 3.53% (RSD = 5.18%) for curdione and 65.04 ± 2.51% (RSD = 3.86%) for curcumol, respectively. The liver probe recoveries were 58.14 ± 2.44% (RSD = 4.19%) for curdione and 60.00 ± 3.49% (RSD = 5.82%) for curcumol, respectively.

#### 3.2 Pharmacokinetic study

The concentration–time profiles for unbound curdione and curcumol in rat blood and liver were shown in Fig. 2. The concentration–time data were analyzed by non-compartmental...
method and the pharmacokinetic parameters were summarized in Table 2.

The double-peak phenomenon of curdione was revealed for the first time. For oral administration, enterohepatic recirculation is the most probable reason for the comparatively small second peak. And it would be expected that a second peak would also occur following intravenous administration (IV) [17]. However, previous studies of curdione indicated that there was no second peak observed in the IV responses [9]. Consequently, enterohepatic recirculation could be ruled out as a possible explanation of the double-peak phenomenon after oral administration of *Rhizoma Curcumae* extract. In our experiment, there are complex components in the extract and some of them have similar structures. Thus some other ingredients might transform into curdione and contribute to the presence of two peaks. However, further studies should be performed to clarify the hypotheses.

The pharmacokinetic profile of curcumin showed that it could be detected both in blood and liver until 1.5 h after oral administration. And the blood concentration rapidly reached a peak at about 2.1 h, while the liver curve had a peak concentration at 2.9 h. The unbound curcumin could be detected for up to 5.5 h. The liver-to-blood distribution ratio of curcumin calculated by dividing the AUC of curcumin in liver by that in blood was 1.07 ± 0.15, which was higher than that of curdione. It was reported that absorption of curcumin from gastrointestinal tract in rats was rapid and complete, which was in accordance with our studies [8]. Curcumin showed poor solubility in water with the structure of a guaiane-type sesquiterpenoid hemiketal [5]. It may be easily metabolized by hepatic microsomal enzyme. Further study is required to investigate the specific mechanisms.

4. Conclusions

The present study is the first investigation on the simultaneous analysis of protein-unbound curdione and curcumin in both rat blood and liver microdialysis samples. The developed RRLC–MS method was validated for all parameters and successfully applied for the pharmacokinetics and liver distribution study in rats. The in vivo microdialysis made it possible for multiple sites sampling in the same individual. It provided the pharmacologically active fraction of curdione and curcumin, which required no further clean-up procedure. In addition, the pharmacokinetic profiles of curdione and curcumin after oral administration of *Rhizoma Curcumae* extracts were illustrated for the first time. Due to the wide use of *Rhizoma Curcumae* in clinic, the established method and our investigations may contribute to further new drug studies of it.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2014.02.025.

References


