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Determination of chlorogenic acid in rat blood by microdialysis coupled with microbore liquid chromatography and its application to pharmacokinetic studies

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Abstract

To investigate the pharmacokinetics of unbound chlorogenic acid, a sensitive microbore liquid chromatographic method for the determination of chlorogenic acid in rat blood by microdialysis has been developed. A microdialysis probe was inserted into the jugular vein of male Sprague–Dawley rats, to which chlorogenic acid (20, 40, 60 or 80 mg/kg, i.v.) had been administered. On-line microdialysate was directly injected into a microbore column using a methanol–100 m*M* sodium dihydrogenphosphate (30:70, v/v, pH 2.5 adjusted with orthophosphoric acid) as the mobile phase and ultraviolet detection at 325 nm. The method is rapid, easily reproduced, selective and sensitive. The limit of detection for chlorogenic acid was 0.01 μg/ml and the limit of quantification was 0.05 μg/ml. The in vivo recovery of the chlorogenic acid of the microdialysis probe, based on a 5 μg/ml standard, was approximately 49–65% ($n=6$). The disposition of chlorogenic acid at each dose was best fitted to a two-compartment pharmacokinetic model. The area under the concentration curve increased greater than in direct proportion with the dose and terminal disposition become much slower as the dose was increased. The results indicated that the pharmacokinetics of unbound chlorogenic acid in rat blood is non-linear. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Microdialysis; Chlorogenic acid; Phenolic compounds

1. Introduction

Chlorogenic acid (3-*O*-ceffeoyl-D-quinic acid; Fig. 1) is an ester formed between caffeic acid and quinic acid, and is one of major polyphenolic compounds found in numerous plant species [1]. Chlorogenic acid has been reported to suppress the *N*-nitrosating reaction [2] and inhibit hepatic glucose 6-phosphatase, which may be a significant factor in the abnormal diabetic state [3]. Moreover, preventive effects of chlorogenic acid in the oxidation [4], lipid peroxidation [5] and hydroxyl free radical [6] have been reported. In view of these important effects, an accurate assay method for the determination of chlorogenic acid in the biological sample is warranted. The most frequently used method for the determination of chlorogenic acid in biological sample is high-performance liquid chromatography (HPLC) [7–9].

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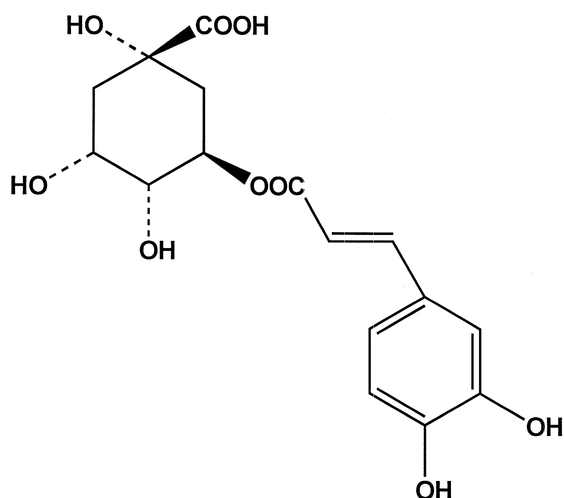

Fig. 1. Chemical structure of chlorogenic acid.

The aim of the present study was to develop a simple, precise, accurate and sensitive microbore HPLC method and couple it to microdialysis for the determination of unbound chlorogenic acid in rat blood. The utility of the method was demonstrated when it was applied to the study of the pharmacokinetics of unbound chlorogenic acid in rat blood.

2. Experimental

2.1. Chemicals and reagents

Chlorogenic acid was purchased from Sigma (St. Louis, MO, USA). Sodium dihydrogenphosphate and HPLC grade reagents were obtained from Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Liquid chromatography

The HPLC system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), an on-line injector (CMA 160, Stockholm, Sweden) equipped with a 10-μl sample loop and a UV detector with micro flow-cell (Soma S-3702 ultraviolet detector, Tokyo, Japan). The detection UV wavelength was set at 325 nm. Chromatography was performed at ambient temperature using a reversed-phase microbore column (BAS, RP-18, 150×1 mm I.D., particle size 5 μm). The mobile phase consisted of methanol–100 m*M* sodium dihydrogenphosphate (30:70, v/v, pH 2.5 adjusted with orthophosphoric acid), pre-filtered through a 0.22-μm Millipore membrane, and was pumped through the system at a flow-rate of 0.05 ml/min. Output data from the detector were integrated via an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.3. Method validation

All calibration curves were required to have a correlation value of at least 0.995. The intra- and inter-day variabilities were determined by quantitating six replicates at concentrations of 0.05, 0.1, 0.5, 1 and 5 μg/ml using the HPLC method described above on the same day and six successive days, respectively. The accuracy was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentrations (C_{obs}) as follows: accuracy $(\%)=[(C_{nom}-C_{obs})/C_{nom}]\cdot 100$. The precision relative standard deviation (RSD) was calculated from the observed concentrations as follows: precision $(\%)=[\text{standard deviation (SD)}/C_{obs}]\cdot 100$ [10,11]. The limit of detection (LOD) is the smallest concentration that can be distinguished from the noise level, at a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) is defined as the lowest concentration on the calibration curve that can be measured with acceptable precision, with a RSD not exceeding 20% [12].

2.4. Microdialysis experiments

Blood dialysis probes were made of silica capillary and were concentric in design. The dialytic surface was provided by covering the ends of the concentric tubing with dialysis membrane (Spectrum, 10 mm×150 μm O.D. with a cut-off at nominal molecular mass of 13 000, Laguna Hills, CA, USA) [13]. The microdialysis probe was inserted into the right jugular vein and perfused with the anticoagulant ACD solution (3.5 m*M* citric acid, 7.5 m*M* sodium citrate, 13.6 m*M* dextrose) at a

flow-rate of 1 μl/min using a CMA/100 microinjection pump.

2.5. In vivo recovery

A retrograde calibration technique was used for the assessment of in vivo recovery. The blood microdialysis probe was inserted into the rat jugular vein under anesthesia with sodium pentobarbital. ACD solution containing chlorogenic acid (5 μg/ml) was passed through the probe at a constant flow-rate (1 μl/min) using a microinjection pump (CMA/100). Two hours post probe implantation, which served as a stabilization period, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of chlorogenic acid were determined by HPLC. The relative loss of chlorogenic acid during retrodialysis L_{retro} or relative recovery (R_{dial}) by dialysis, was then calculated as follows [14]: $L_{retro}=R_{dial}=(C_{perf}-C_{dial})/C_{perf}$.

2.6. Pharmacokinetics study

Experiments were performed on adult male specific pathogen-free Sprague–Dawley rats (280–320 g) obtained from the Laboratory Animal Center at National Yang-Ming University (Taipei, Taiwan). The rats were acclimated in their environmentally controlled quarters (24±1°C and 12:12 h light–dark cycle) for at least 5 days before being used in experiments. On the day of experiment, rat was initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and catheters were placed in the femoral vein and jugular vein for drug administration and dialysis, respectively. The rat's body temperature was maintained at 37°C with a heating blanket. Following a 2-h stabilization period after surgery, chlorogenic acid was examined after intravenous bolus administration in individual rat for each of the doses tested (20, 40, 60 or 80 mg/kg, $n=6$ for each doses). Dialysates were injected every 10 min by an on-line injector (CMA/160) for an additional 2 to 6 h following chlorogenic acid administration. Chlorogenic acid microdialysis concentrations (C_m) were converted to unbound concentration (C_u) as follows [14]: $C_u=C_m/L_{retro}$.

Pharmacokinetic calculations were performed on each individual set of data using the pharmacokinetic software WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA). The compartmental model could be a sum of exponential of the follow: $C=\Sigma C_i \cdot \exp(-\lambda_i t)$. Where C is the predicted concentration, t is time, C_i (=A, B, ...) and λ_i (=α, β, ...) are the pre-exponential and exponential coefficients, was fitted to blood concentration data using nonlinear regression analysis. The areas under the concentration–time curves (AUCs) were calculated by the linear trapezoid rule up to the last detectable concentration and extrapolated to infinite time using the terminal elimination rate; analogous method was used for the calculation of the area under the first moment curve (AUMC), but using the concentration vs. time data. Half-life ($t_{1/2}$) values were calculated using the equations: $t_{1/2,\ \lambda_i}=0.693/\lambda_i$. Mean residence time (MRT) was calculated as AUMC/AUC. Volume of distribution (Vc) of the central compartment was calculated as dose/C_0, where C_0 is the concentration measured just after the administration. Clearance (Cl) was calculated as dose/AUC [15].

Comparisons of pharmacokinetic parameters between doses were performed using t-tests for paired observations. A P value of <0.05 was considered significant. All data are presented as means±standard errors.

3. Results and discussion

3.1. Specificity of chlorogenic acid in blood microdialysate

The present microbore liquid chromatographic method was coupled to the microdialysis technique and applied to the determination of chlorogenic acid from rat jugular vein following drug administration. Under the conditions described above, the retention time of chlorogenic acid was found to be 5.1 min (Fig. 2). Fig. 2A shows a standard injection of chlorogenic acid (0.5 μg/ml). Fig. 2B shows a chromatogram of a blank blood dialysate. No peaks were observed that would interfere with the analysis of either compound. Fig. 2C shows a chromatogram of a blood dialysate sample containing chlorogenic acid (0.12 μg/ml) obtained from blood microdialysis

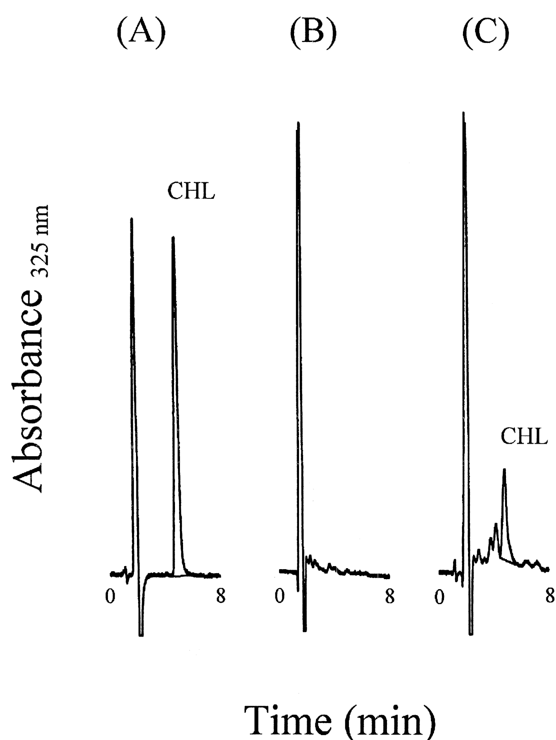

Fig. 2. Typical chromatogram of injection of (A) standard chlorogenic acid (CHL; at 0.5 μg/ml), (B) a blank blood dialysate, and (C) a blood dialysate sample containing chlorogenic acid (0.12 μg/ml) collected from jugular vein at 140 min after chlorogenic acid administration (60 mg/kg, i.v.).

140 min after chlorogenic acid administration (60 mg/kg, i.v.).

3.2. Linearity, precision and accuracy

The method was linear ($r^2>0.995$) for chlorogenic acid over a concentration range of 0.05–10 μg/ml. Intra- and inter-day precision and accuracy for chlorogenic acid (Table 1) fell well within pre-defined limits of acceptability. All % bias and RSD values were within ±10%. The LOD and LOQ for chlorogenic acid were 0.01 μg/ml and 0.05 μg/ml, respectively, within the pre-defined level [12].

3.3. Microdialysis and pharmacokinetics

The in vivo recovery of chlorogenic acid was 60.37±5.97% (based on 5 μg/ml of standard) as shown in Table 2. The concentration of unbound chlorogenic acid in rat blood after chlorogenic acid (20, 40, 60 or 80 mg/kg, i.v.) administrations are shown in Fig. 3. A two-compartment model was proposed and validated through the program to explain the apparent bi-phasic disposition of chlorogenic acid in rat blood after an intravenous bolus injection. The dialysate samples collected over the first 2 h were discarded to allow recovery from the acute effects of the surgical procedure. The microdialysis sampling technique and liquid chromato-

Table 1
Intra- and inter-assay variabilities for chlorogenic acid

Nominal concentration (μg/ml)	Observed concentration (μg/ml)[a]	RSD (%)	Accuracy (% bias)
Intra-assay (n=6)			
0.05	0.051±0.002	3.9	2.0
0.1	0.099±0.007	7.1	−1.0
0.5	0.49±0.016	3.3	−2.0
1	0.98±0.023	2.3	−2.0
5	4.99±0.008	0.2	−0.2
Inter-assay (n=4)			
0.05	0.055±0.005	9.1	10.0
0.1	0.11±0.004	3.6	10.0
0.5	0.49±0.003	0.6	−2.0
1	0.98±0.011	1.1	−2.0
5	5.02±0.026	0.5	0.4

[a] Observed concentration data are expressed as rounded means±SD.

Table 2
Retrograde in vivo microdialysis recovery of chlorogenic acid (based on 5 μg/ml) at infusion rate of 1 μl/min

Animal No.	Recovery (%)
Rat 1	49.39
Rat 2	58.42
Rat 3	61.47
Rat 4	62.44
Rat 5	64.68
Rat 6	65.82
Mean±SD	60.37±5.97

graphic detection were then applied to the pharmacokinetic characterization of chlorogenic acid in rats (Table 3).

When the dose of chlorogenic acid was increased from 20 to 80 mg/kg, AUC and AUMC in rats increased greater than in direct proportion with the dose. In addition, the terminal half-life ($t_{1/2,\ \beta}$) was longer at higher doses. These results suggest that the pharmacokinetics of chlorogenic acid in rats is non-linear. The same non-linear pharmacokinetics phenomena were observed that in some other ingredients of commonly used herbal medicine such as glycyrrhizin [16], asarone [17], and glycyrrhetinic acid [18] in our previous studies. In cases like these, administration of a large dose may lead to retardation of the drug elimination and prolongation of its effect [15]. In contract, magnolol [19] exhibits linear pharmacokinetics.

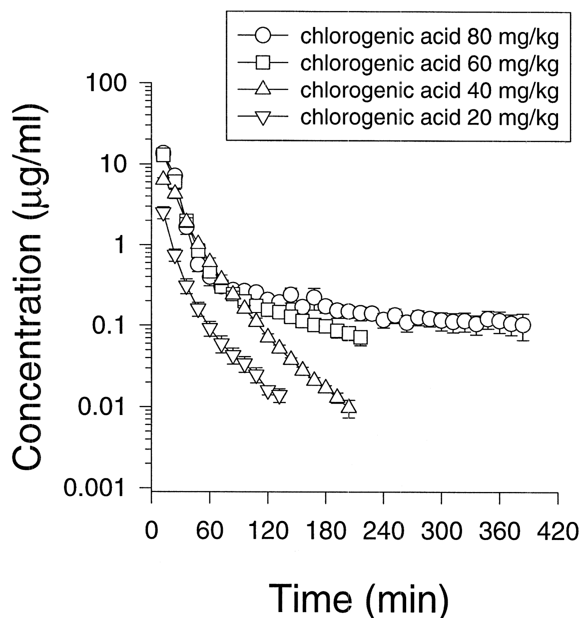

Fig. 3. Mean concentration of chlorogenic acid in the rat blood after chlorogenic acid administration (20, 40, 60 and 80 mg/kg, i.v.).

The present on-line microdialysis technique provides protein-free samples that can be automatically injected into a liquid chromatographic system for continuous in vivo monitoring of unbound drugs in blood. Further, as this sampling method involves virtually no loss of body fluids, it facilitates pharmacokinetics studies by eliminating the effects of blood volume changes as compared to conventional blood withdrawal designs [20]. In addition, microdialysis is relatively inexpensive and easy to set up. Laboratory-made probes further reduce expenses and allow customization for special needs such as sampling in blood. With certain precautions, such as allowing time for stabilization and partial recovery from surgical trauma and careful recovery calibration, the disadvantages can be minimized. The dialysates can be analyzed using any appropriate analytical methods. However, sensitivity of the method dictates the amount of dialysate required and therefore the sampling time, hence a disadvantage in that the values actually represent mean values over the period of dialysate collection. In our case, microbore HPLC is again relatively accessible and the method has been optimized so that only a 10 min collection time is required.

4. Conclusion

This microdialysis technique provides protein-free samples that can be directly injected into a liquid chromatographic system for continuous in vivo monitoring of unbound drugs in blood. Further, this sampling method facilitates pharmacokinetic studies by eliminating the effects of blood volume changes as compared to conventional blood withdrawal designs. The results demonstrate that the pharmacokinetics of unbound chlorogenic acid in rat blood is non-linear.

Table 3
Pharmacokinetic parameters following chlorogenic acid administration (20, 40, 60 and 80 mg/kg, i.v.; data are expressed as mean±S.E.M., $n=6$)

Parameter	Administration (mg/kg)			
	20	40	60	80
A (μg/ml)	8.35±2.26	11.54±2.45	37.49±4.45[b]	74.51±22.68[c]
B (μg/ml)	0.57±0.24	1.37±0.72	0.41±0.09	0.39±0.069
α (1/min)	0.14±0.016	0.067±0.015	0.11±0.01	0.13±0.0093
β (1/min)	0.031±0.006	0.039±0.0069	0.01±0.0021	0.0061±0.001
$t_{1/2,\beta}$ (min)	21.16±2.60	23.22±6.80	88.05±20.64[b]	172.37±57.36[c]
AUC (μg min/ml)	73±13	211±13[a]	389±2[b]	623±135[c]
Vc (l)	2.91±0.65	3.28±0.36	1.74±0.27	1.61±0.44
Cl (l/min/kg)	0.31±0.048	0.19±0.012[a]	0.16±0.01[b]	0.16±0.042
AUMC (μg min^2/ml)	916±122	3867±416[a]	7572±1164[b]	15 342±3588[c]
MRT (min)	13.73±2.67	18.11±1.46	19.52±2.83	25.01±2.07

[a] Significantly different ($P<0.05$) from the dose of 20 mg/kg.
[b] Significantly different ($P<0.05$) from the doses of 20 and 40 mg/kg.
[c] Significantly different ($P<0.05$) from the dose of 20, 40 and 60 mg/kg.

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